Presence of Environmental *Burkholderia pseudomallei* and Burden of Melioidosis in Thailand

Thesis

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Presence of environmental *Burkholderia pseudomallei* and burden of melioidosis in Thailand

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Abstract

Burkholderia pseudomallei is a soil-dwelling bacterium and the cause of melioidosis. Currently knowledge of the distribution of environmental *B. pseudomallei* and of the factors determining it is limited. In Thailand *B. thailandensis*, a closely related species to *B. pseudomallei*, is common, though the implications for *B. pseudomallei* distribution is unknown. Due to the difficulties in diagnosis of melioidosis and lack of resources, the distribution and burden of human melioidosis is likely under-reported. Melioidosis is a notifiable disease in Thailand. It has been estimated that more than 2,000 deaths are caused by melioidosis in Thailand, but only around 10 melioidosis deaths were officially reported to the notifiable diseases surveillance system (Report 506) each year.

This thesis describes two large epidemiological studies: an environmental survey; and a cross-sectional retrospective, multicenter surveillance study using data from hospital databases nationwide. The environmental survey examined the distribution of environmental *B. pseudomallei* and *B. thailandensis*, ecological factors, and the seropositivity of farmers working in the fields. The retrospective surveillance study determined the incidence and mortality of human melioidosis diagnosed in large public hospitals throughout Thailand.

I found that *B. pseudomallei* is widely distributed in East and Northeast Thailand, and unevenly distributed in Central Thailand. *B. pseudomallei* thrives in rice fields that are nutrient depleted. Presence of *B. pseudomallei* and *B. thailandensis* in the same field is not uncommon. In addition, *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide (BTCV) was isolated from soil for the first time in Thailand. Background seropositivity against *B. pseudomallei* of healthy rice farmers in Thailand is associated with

presence of *B. pseudomallei* in rice fields rather than *B. thailandensis* or BTCV. Finally, melioidosis is endemic and is an important cause of death in Thailand, but is rarely officially reported to the Thai Ministry of Public Health. Data from the national notifiable disease-surveillance system in resource-limited settings should be verified by integrating information from readily available databases.

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List of Abbreviations

AvailMg	available magnesium
AvailP	available phosphorous
BODCVS	Bureau of Disease Control and Veterinary Services
BOE	Bureau of Epidemiology
B. ma	*B. mallei*
B. ps	*B. pseudomallei*
BTCV	*B. thailandensis* expressing *B. pseudomallei*-like capsularpolysaccharide
B. th	*B. thailandensis*
C:N ratio	carbon to nitrogen ratio
CD	total cadmium
CDC	Centers for Disease Control
CFR	case fatality rate
CFT	complement fixation test
CI	confidence interval
cmol/mg	centimoles per milligram
COD	carbon oxygen demand
CPS	capsular polysaccharide
DEBWorP	Detection of Environmental *Burkholderia pseudomallei* Working Party
DHO	district health office
DNA	Deoxyribonucleic acid
dS/m	deciSiements/meter
EC	electrical conductivity level
ELISA	enzyme-linked immunosorbent assay
ExchCa	exchangeable calcium
ExchK	exchangeable potassium
ExtrS	extracable sulphur
Fe	Iron
g/kg	gram per kilogram
IFA	immunogfluorescence assay

IgG	immunoglobulin G
IgM	immunoglobulin M
IHA	Indirect Haemagglutination Assay
IQR	interquartile range
kg/100sqm	Kilogram per 100 sqaure meters
LDD	Land Development Department
LFI	lateral flow immunoassay
LR	lime requirement
mg/kg	milligram per kilogram
MLST	multilocus sequence typing
MoPH	Ministry of Public Health
MUTM	Faculty of Tropical Medicine, Mahidol University
OM	organic matter
OR	odds ratio
OXTREC	Oxford Tropical Research Ethics Committee
PCD	Pollution Control Department
PCR	polymerase chain reaction
PPHO	provincial public health office
RNA	ribonucleic acid
ST	sequence type
TBSS	threonine-basal salt solution
TotalN	total nitrogen
TTSS	type III secretion system
VBNC	viable but non-culturable state
WHO	World Health Organization
%w/w	% weight for weight
$\mu W/cm^2$	micro-watts per square centimeter

Publication arising from this thesis

1. **Hantrakun V**, Rongkard P, Oyuchua M, Amornchai P, Lim C, Wuthiekanun V, Day NP, Peacock SJ, Limmathurotsakul D: Soil nutrient depletion is associated with the presence of *Burkholderia pseudomallei*. Appl Environ Microbiol 2016, 82:7086-7092
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Sachaphimukh S, Wannapinij P, Chierakul W, Chewapreecha C, Thaipadungpanit J, Chantratita N, Korbsrisate S, Taunyok A, Dunachie S, Palittapongarnpim P, Sirisinha S, Kitphati R, Iamsirithaworn S, Chaowagul W, Chetchotisak P, Whistler T, Wongratanacheewin S, Limmathurotsakul D: Melioidosis in Thailand: Present and Future. Trop Med Infect Dis. 2018;3(2):38.

5. **Hantrakun V,** Kongyu S, Klaytong P, Rongsumlee S, Smithsuwan P, Kitphati R, Day NP, Peacock SJ, Hinjoy S, Limmathurotsakul D: Supplementing national notifiable diseases surveillance data with routinely available databases: an example of melioidosis in a developing country (manuscript in preparation)

Chapter 1: Introduction and scope of the thesis

1.1 A brief history of melioidosis

Over a hundred years ago, in 1912, the pathologist Alfred Whitmore and his assistant C. S. Krishnaswami described "a strange infective diseases somewhat resembling glanders", which took 38 lives in Rangoon, Burma (now Myanmar) (Whitemore and Krishnaswami, 1912). A new pathogenic organism was identified during their post-mortem investigations. The morphology of organism was very similar to *Bacillus mallei* (now *Burkholderia mallei*), which is the causative organism of glanders. However, the organism was different by its relatively rapid growth, its motility and the lack of Strauss reaction when injected into guinea pigs. The organism was named "*Bacillus pseudomallei*" (Whitemore and Krishnaswami, 1912). In 1913, Fletcher observed a similar infectious disease among his laboratory animals in Kuala Lumpur, Malaysia (Rush and Thomas, 2012c). In 1921, Stanton and Fletcher published a paper and called this strange infectious disease; "Melioidosis, a new disease of the tropics" (Stanton and Fletcher, 1921). Nonetheless, in the twenties, the epidemiology of melioidosis was relatively unknown.

In 1955, Chambon suggested that soil contaminated with *Bacillus pseudomallei* could be the source of infection. In 1966, *Bacillus pseudomallei* was classified to the genus *Pseudomonas* due to its ability to oxidize carbohydrates. The organism was then called "*Pseudomonas pseudomallei*" (Stanier et al., 1966). In the late sixties, *P. pseudomallei* was frequently isolated from environmental soil and water (Ellison et al., 1969, Strauss et al., 1969). This suggested that the natural habitat of *P. pseudomallei* is soil and surface water. During the Vietnam War, inhalation of *P. pseudomallei* contaminated dust was suspected to be a route of infection among American helicopter crewmen (Howe et al., 1971). This

expanded our understanding that humans may acquire melioidosis following bacterial inoculation, ingestion or inhalation.

In 1992, using DNA and RNA information, *P. pseudomallei* was reclassified to the genus *Burkholderia* by Yabuuchi et al (Yabuuchi et al., 1992). Since then, the causative bacterium of melioidosis has been called *Burkholderia pseudomallei*.

1.2 Microbiology of *B. pseudomallei* and others related species

B. pseudomallei is a Gram-negative, motile, non-sporing forming, and facultative anaerobic bacillus (Rush and Thomas, 2012b). It is an environmental saprophyte, which is free-living in the soil and water in tropical regions. The organism can cause an infectious disease called melioidosis in both humans and animals. The bacterium is also known for its remarkable ability to survive under harsh environment, including in an environment without nutrients (Wuthiekanun et al., 1995b, Moore et al., 2008).

1.2.1 Colony morphology

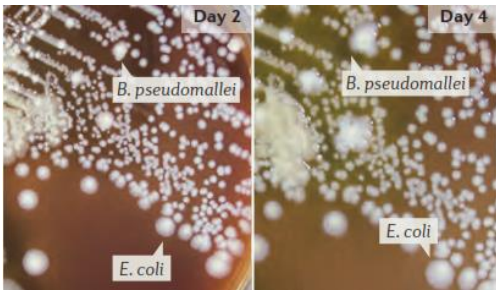
B. pseudomallei can grow on common culture media (e.g. sheep blood agar and MacConkey agar), and in mixed culture it may grow slower but can be distinguished from other organisms by use of selective agars (e.g. Ashdown agar and commercial *Burkholderia cepacia* medium). Its colonies may have different morphologies on different types of agar, and these may change over the period of incubation. After 48 hours of incubation, colony appearances could be creamy-white to grey, colorless of non-lactose fermenter, and pink color on sheep blood agar, MacConkey agar, and Ashdown agar, respectively (Figure 1-1). After 4 days of incubation, colonies glisten with a metallic sheen (on sheep blood agar), become pale pink and may have a sheen (on MacConkey agar), and change from pink to purple with a metallic sheen (on Ashdown agar). The most characteristic feature of *B.*

pseudomallei is its metallic sheen, and the usual progression to dry and wrinkled colonies. (Figure 1-1).

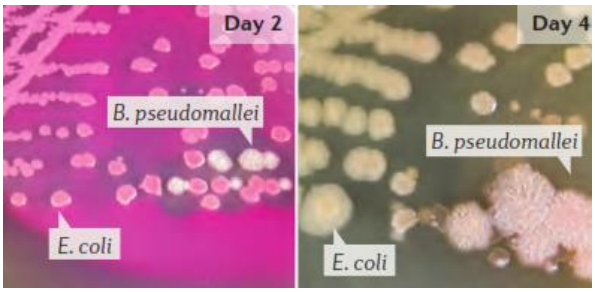
Ashdown agar is the most commonly used selective medium, and on it clinical isolates of *B. pseudomallei* can have one of seven types of colony morphology (categorized as Type I to Type VII). Type I is the classical colony morphology, pale purple with a wrinkled outer edge (Chantratita et al., 2007a) (Figure 1-2). Environmental isolates of *B. pseudomallei* are reported to have common colony morphologies similar to those of the clinical isolates (Smith et al., 1997). However, *B. pseudomallei* colonies are indistinguishable from a closely-related nonpathogenic species, *Burkholderia thailandensis* (Smith et al., 1997). Therefore, definite identification of *B. pseudomallei* requires additional tests including biochemical tests, the latex agglutination test and antimicrobial susceptibility testing (described in following diagnostics section 1.3.4).

Figure 1-1 Colony morphologies of *B. pseudomallei* after incubation for 2 days and 4 days on (a) sheep blood agar, (b) MacConkey agar, and (c) Ashdown agar described previously by Wiersinga et al (Wiersinga et al., 2018).

a. Sheep blood agar



b. MacConkey agar



c. Ashdown agar

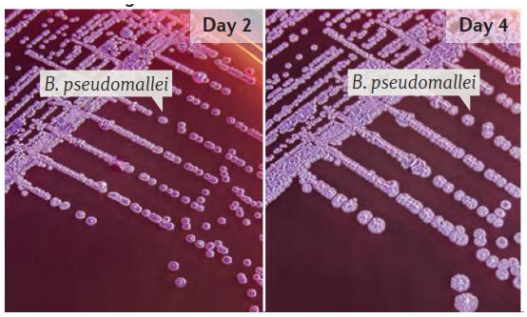
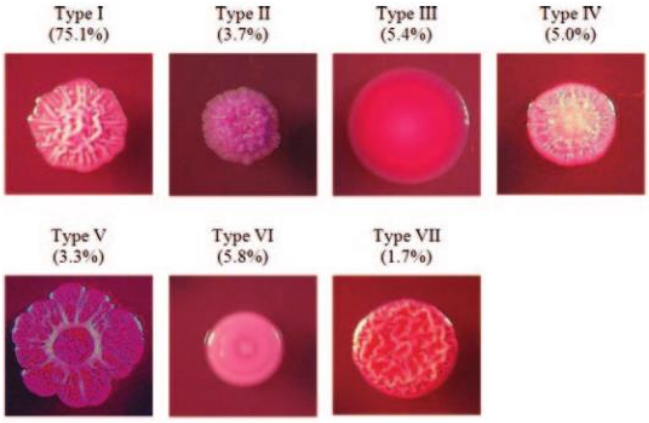


Figure 1-2 Seven types of colony morphology of *B. pseudomallei* on Ashdown agar described previously by Chantratita et al 2007 (Chantratita et al., 2007a)



1.2.2 Other related species in the *Burkholderia* genus

B. pseudomallei belongs to the *Burkholderia* genus, which contains over 40 species. Other notable species include the pathogenic species *B. mallei* and *B. cepacia*, and the non-pathogenic species *B. ubonensis and B. thailandensis*.

1.2.2.1 *B. mallei*

B. mallei is the causative organism of glanders. Glanders is an infectious disease that occurs primarily in horses, mules and donkeys. Glanders is a communicable disease, which can be transmitted from an infected animal to other animals and to human. The transmission routes include an exposure to the discharges of infected animals, inhalation of contaminated aerosols (respiratory inoculation), and consumption of infected animal meat. Clinical manifestations of glanders in infected animals include necrotic ulcers and nodules in nasal passages, enlarged mediastinal lymph nodes, and pneumonia with nodular abscesses. Human infection presents in a similar way to equine infection, with manifestations including pneumonia, pleural nodules, regional lymphadenopathy and abscesses in the liver and spleen. However, both animal and human glanders is now rare due to better prevention and control, and implementation of multi-national glanders eradication programmes. Sporadic animal glanders are still reported in the Middle East (Scholz et al., 2014), Asia (Saqib et al., 2012), and South America (Mota et al., 2010).

A genetic study concluded that *B. mallei* evolved from *B. pseudomallei* by gene reduction (Godoy et al., 2003, Nierman et al., 2004). Unlike *B. pseudomallei*, *B. mallei* is an obligate pathogen which does not exist in the environment.

1.2.2.2 *B. cepacia*

B. cepacia is another pathogenic species which causes opportunistic infections, most notably in patients with cystic fibrosis. *B. cepacia* is also an emerging cause of hospital acquired infection. The bacterium can be found in the natural environment such as soil and water (LiPuma et al., 2002, Dalmastri et al., 1999, Ibrahim et al., 2012, Butler et al., 1995). In the 1990s, *B. cepacia* was developed to be a bio-pesticide protecting crops against fungal diseases (Holmes et al., 1998). Later, the organism became widely acknowledged as an opportunistic pathogen causing infections in cystic fibrosis patients (Holmes et al., 1998, Tanser et al., 2000, Cipolla et al., 2017). The route of infection include exposure to the organism via person-to-person contact, and contact with contaminated surfaces and environment (Humphreys et al., 1994).

1.2.2.3 *B. ubonensis*

B. ubonensis is a non-pathogenic saprophyte which can be isolated from soil and water (Yabuuchi et al., 2000, Price et al., 2017). A recent report demonstrated that *B. ubonensis* is avirulent in a mouse model (Price et al., 2017).

1.2.2.4 *B. thailandensis*

B. thailandensis is found in soil and water, and was first recognized by Wuthiekanun et al. in 1996 (Wuthiekanun et al., 1996). The organism is genetically closely related to *B. pseudomallei*; however, it is non-pathogenic (Wuthiekanun et al., 1996, Trakulsomboon et al., 1997, Sermswan et al., 2015). The colony morphologies of *B. thailandensis* and *B. pseudomallei* are very similar. *B. thailandensis* can be differentiated from *B. pseudomallei* by its ability to assimilate L-arabinose (Smith et al., 1997, Brett et al., 1998). The genomic study shows that an arabinose assimilation operon (BTH_II1626–1633) on Chromosome 2

is present in *B. thailandensis* but absent in *B. pseudomallei* (Yu et al., 2006). In addition, *B. thailandensis* has polysaccharide-related genes that are distinct from *B. pseudomallei* (74.8% and 72.8% nucleotide and protein similarity, respectively) and usually lacks the virulence-associated capsular polysaccharide (also referred to as CPS or CPS-I) of *B. pseudomallei* (Reckseidler et al., 2001, Smith et al., 1993, Wuthiekanun et al., 2002, Sim et al., 2010). *B. thailandensis* also lacks a gene cluster BPSL2790-2810, which is considered a major determinant of virulence in *B. pseudomallei.* The gene cluster BSP2790-2810 is involved in the synthesis and export of the capsular polysaccharide (Reckseidler et al., 2001).

A variant of *B. thailandensis* that contains genes encoding a *B. pseudomallei*-like capsular polysaccharide cluster (BTCV) was isolated from soil in Cambodia (strain E555; ST696) (Sim et al., 2010). This organism exhibits several *B. pseudomallei*-like phenotypes including colony wrinkling, resistance to human complement binding, and intracellular macrophage survival. In a mouse model, E555 is avirulent (Sim et al., 2010), induces higher levels of IgG and gives better protection against melioidosis than non-capsulated *B. thailandensis* (Scott et al., 2013). The capsular polysaccharide (CPS) biosynthesis gene cluster of E555 and that of *B. pseudomallei* are highly similar (94.4% and 96% nucleotide and protein similarity, respectively) (Sim et al., 2010), and nuclear magnetic resonance spectroscopy has shown that the structures of CPS produced by E555 and that of *B. pseudomallei* are identical (Bayliss et al., 2017). In addition, this variant of *B. thailandensis* (BTCV) has been isolated from human blood in the USA (strain CDC3015869; ST101, USA (Glass et al., 2006)) and from environmental samples in Gabon (strain D50; ST1126

(Wiersinga et al., 2015)) and Laos (strain ST_10; ST696 (Knappik et al., 2015)). BTCV has not been reported in Thailand, and its full distribution is unknown.

The geographical distribution of *B. thailandensis* is uncertain but the organism has rarely been isolated from fields that are culture positive for *B. pseudomallei* (Vuddhakul et al., 1999, Trakulsomboon et al., 1999). It was recently shown that *B. pseudomallei* can inhibit the growth and motility of *B. thailandensis* in the laboratory (Ngamdee et al., 2015). However, previous environmental studies did not systematically evaluate the presence of both organisms, so the presence of *B. thailandensis* and co-localization of both organisms may have been underestimated (Vuddhakul et al., 1999, Trakulsomboon et al., 1999). In an experimental mouse model, lipopolysaccharide extracted from *B. thailandensis* induced measurable IgG and IgM, and provided partial protection against melioidosis (Ngugi et al., 2010). The association between exposure to environmental *B. thailandensis* and seropositivity to *B. pseudomallei* as measured by the indirect hemagglutination assay (IHA) in humans is still largely unknown.

1.2.3 Virulence determinants in *B. pseudomallei*

Multiple factors are associated with the virulence of *B. pseudomallei*. Humans may acquire *B. pseudomallei* via percutaneous inoculation, inhalation, aspiration or ingestion. *B. pseudomallei* infection may start from attaching to and passing into human epithelial cells (i.e. mucosal surface or broken skin), and then replicating inside those cells (Lazar Adler et al., 2009). Bacterial surface components including flagella, capsular polysaccharide and type IV pili (hair-like structures on the bacterial surface) facilitate the close contact and attachment to epithelial cells (Ahmed et al., 1999). *B. pseudomallei* can also replicate and survive within many eukaryotic cell lines, including professional phagocytes such as

neutrophils and macrophages. Important bacterial virulence factors of *B. pseudomallei* include the type III secretion system, capsular polysaccharide, lipopolysaccharide and quorum sensing.

1.2.3.1 Type III secretion system (TTSS)

B. pseudomallei contains three Type III secretion system gene clusters, TTSS-1, TTSS-2 and TTSS-3 (Holden et al., 2004), that play an important role in virulence. A gene cluster in *B. pseudomallei* called *bsa* (*Burkholderia* secretion apparatus)-encoded TTSS is essential for invading cells, escaping from endocytic vacuoles, replicating inside host cells (Stevens et al., 2002, Stevens et al., 2003, Stevens et al., 2004), forming multinucleated giant cells, and inducing apoptosis of infected host cell (Suparak et al., 2005). Specifically, TTSS-3 is required for full virulence in a hamster infection model (Warawa and Woods, 2005). TTSS-3 encodes a secretion apparatus that injects bacterial effector proteins into the host-cell cytoplasm and enhance cell invasion (Cornelis and Van Gijsegem, 2000).

A genetic study comparing between *B. pseudomallei*, *B. mallei* and *B. thailandensis* shows that the TTSS1 cluster in *B. pseudomallei* is not present in either *B. mallei* or *B. thailandensis* (Kim et al., 2005) (Figure 1-3 panel C). Thus, TTSS1 is used as a target for the molecular identification of *B. pseudomallei* (Novak et al., 2006).

1.2.3.2 Capsular polysaccharide (CPS)

CPS has been shown to be a virulence factor in other organisms through a number of mechanisms such as adherence for colonization, resistance to complement mediated phagocytosis and killing, and resistance to specific host immunity. *B. pseudomallei* possess CPS with structure -3)-2-O-acetyl-6-de-oxy-b-D-manno-heptopyranose-, which has been previously characterized as type I O-polysacharide (OPS) (Perry et al., 1995, Reckseidler et

al., 2001). A study using a Syrian hamster model shows that this CPS is required for *B. pseudomallei* infection (Reckseidler et al., 2001). A subsequent experiment using the serum bactericidal assay shows that the CPS contributes to resistance to complement mediated phagocytosis (Reckseidler-Zenteno et al., 2005). CPS may be involved in epithelial adherence because it mediates attachment of bacteria to pharyngeal epithelial cells (Ahmed et al., 1999).

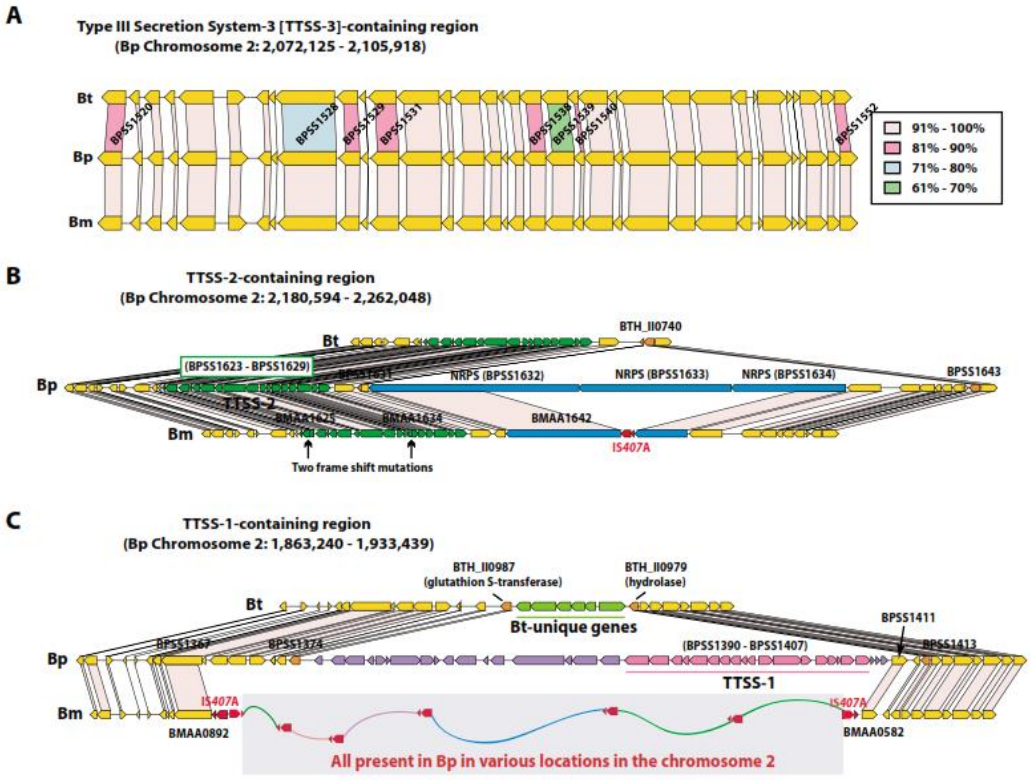
CPS gene clusters are present in *B. pseudomallei* and *B. mallei* but not in *B. thailandensis* (Sim et al., 2010, Kim et al., 2005). A genomic study showed that this CPS gene cluster is also present in environmental *B. thailandensis* strain E555; *B. thailandensis* expressing *B. pseudomallei*-like CPS variant (BTCV). However, BTCV is not virulent in a mouse model (Sim et al., 2010).

1.2.3.3 Lipopolysaccharide (LPS)

LPS extends from the outer membrane of Gram-negative bacterium, and plays important roles in providing a strong barrier against toxic compounds such as antibiotics, and enhancing survival in harsh environments. The Lipid A component may assist *B. pseudomallei* to avoid recognition by the host immune system (such as through Toll-like receptors) and enhances its survival in host cells (DeShazer et al., 1998, Burtnick and Woods, 1999, Arjcharoen et al., 2007, Wiersinga et al., 2007).

Figure 1-3 Comparison of the Type III secretion Systems (TTSSs) and the surrounding regions in *B. pseudomallei* (*B. ps*), *B. mallei* (*B. ma*), and *B. thailandensis* (*B. th*) by Kim et al 2005 (Kim et al., 2005)

The orthologous genes in the three species are denoted with connecting lines.(A) Comparison of TTSS-3 locus among the three species. The % amino acid identity was determined using TBLASTN from *B. ps* proteins, and is color coded accordingly. (B) Comparison of TTSS-2 and its vicinity. Large deletions in the genes coding for non ribosomal peptide syn-thases (NRPSs) in *B. ma* and *B. th* are shown. Two frame shift mutations in the two genes in the TTSS-2 of *B. ma* also are shown. (C) Comparison of the regions around TTSS-1. The fragment containing TTSS-1 and the surrounding genes that are only present in *B. ps*, and the replacement fragments of this in *B. th* and *B. ma* are shown.



1.2.3.4 Quorum sensing (QS)

QS is an intercellular (cell-to-cell) communication system in Gram-negative bacteria. QS produces and detects diffusible signalling molecules – acylated homoserine lactones (AHLs) – which leads to changes in gene expression (Lazdunski et al., 2004, Ulrich et al., 2004). In a Syrian hamster model, *B. pseudomallei* with *luxl* mutations (especially *bpmI1*, *bpmI3*) showed increased time to death and reduced lung colonization in an aerosolized

BALB/c mice model, indicating that QS networks is essential for *B. pseudomallei* pathogenicity (Ulrich et al., 2004).

1.3 Epidemiology of human melioidosis

Melioidosis is a serious community-acquired infectious disease caused by *B. pseudomallei*. The distribution of human melioidosis, risk factors for infection, clinical manifestations, diagnosis and identification of *B. pseudomallei*, and treatment are described in this section.

1.3.1 Worldwide distribution of human melioidosis

Northeast Thailand and the most northern regions of Australia are known as highly endemic areas for melioidosis, with annual incidence rates of up to 50 cases per 100,000 people (Limmathurotsakul et al., 2010b, Currie et al., 2010). A number of countries in East and South Asia are also endemic areas for the disease, including China (including Southern China, Taiwan and Hong Kong), Cambodia, Indonesia, Brunei, Malaysia, Lao PDR, Myanmar, Vietnam, Philippines, and Singapore. In recent years, there has been an increase in the reporting of melioidosis cases from South Asian countries (Bangladesh, India, Nepal, Pakistan, Bhutan, Maldives and Sri Lanka), and African countries (Burkina Faso, Gabon, Gambia, Kenya, Madagascar, Mauritius, Malawi, Nigeria, and Uganda) (Figure 1-4).

Sporadic cases of melioidosis have been reported from Western Asia, the Middle East, and the Americas. The reporting countries in Western Asia and the Middle East are Turkey and Iran. Countries or islands in the Americas reporting melioidosis cases include Costa Rica, Peru, Brazil, Colombia, Dominican Republic, Ecuador, Guatemala, Honduras, Mexico, Panama, Puerto Rico, Aruba, Martinique, British Virgin Islands, and Venezuela (Figure 1-4). The majority of human cases in Central and South Americas are speculated to

be indigenous melioidosis, because environmental *B. pseudomallei* is also found in the region (Benoit et al., 2015a).

Melioidosis is not indigenous in European countries, Canada and the United States of America (USA). Melioidosis cases reported in those countries were returned-travelers who acquired the infection from endemic regions, and their clinical symptoms appeared after their return home (Dan, 2015, Benoit et al., 2015a). There have been several cases reported from Canada and the USA of laboratory acquired melioidosis (Green and Tuffnell, 1968, Schlech et al., 1981, Benoit et al., 2015b).

Figure 1-4 Published distribution of human melioidosis (www.melioidosis.info)

Despite the recent increase in publications of melioidosis cases, the global distribution of melioidosis is still largely unclear. A recent modelling study predicted 165,000 (95% credible interval 68,000 – 412,000) human melioidosis cases per year worldwide, with a predicted 89,000 (36,000-227,000) deaths (Limmathurotsakul et al., 2016). Modelling environmental factors, the same study also predicted that the highest incidence of melioidosis would occur in South Asia, followed by East Asia and the Pacific, Sub-Saharan

Africa, Latin America and Caribean, and Middle East and North Africa (Limmathurotsakul et al., 2016). The study predicted that melioidosis is not present in Europe, Central Asia, and North America because their environment is not suitable for *B. pseudomallei* (Limmathurotsakul et al., 2016).

1.3.2 Risk factors

The most important risk factor for melioidosis is diabetes mellitus, which is present in half of culture-confirmed melioidosis patients (White, 2003, Limmathurotsakul and Peacock, 2011). Having diabetes mellitus increases the odds of having *B. pseudomallei* infection about 10 times more than control groups of non-infected controls and patients with bacteraemia caused by other organisms (Suputtamongkol et al., 1999). Other clinical risk factors for melioidosis include chronic kidney disease, chronic lung disease, rheumatic heart disease/congestive heart failure, malignancy, immunosuppressive therapy (including steroid use) and other immunosuppression – but not HIV (Limmathurotsakul et al., 2006a, Currie et al., 2010). These risk factors may represent an impairment of the ability of neutrophils and other phagocytic cells to attenuate or kill the pathogenic organism once inside host cells (Easton et al., 2007). In addition to host risk factors, climatic factors (Kaestli et al., 2016), daily living activities including rice farming and exposure to soil and environmental water (Limmathurotsakul et al., 2013b), and occupational activities, such as accidental laboratory exposure, are also associated with an increased risk of acquiring the infection. A rise in the dew point, cloud cover, rainfall, maximum temperature and groundwater could contribute to the risk of infection (Kaestli et al., 2016).

Despite melioidosis being prominent in people age from 40 to 60 years, this infectious disease occurs at all ages. About 20% of all melioidosis cases in adults have no known

clinical risk factors (Currie et al., 2010, Currie et al., 2004, Limmathurotsakul et al., 2010b). Unlike melioidosis in adults, clinical risk factors are not commonly observed in pediatric cases (Turner et al., 2016, Edmond et al., 1998, Lumbiganon and Viengnondha, 1995).

1.3.3 Clinical presentations

Severity and outcome of melioidosis relate to acquired bacterial load, host risk factors and the putative variation in virulence of *B. pseudomallei* strains. An exposure which leads to acquisition of the pathogen could be via occupational or daily living activities (Limmathurotsakul et al., 2013b), though not all exposures to the pathogen lead to infection (Limmathurotsakul and Peacock, 2011). Little is known about the magnitude (e.g. frequency and durations) of exposure to environmental *B. pseudomallei* that could lead to infection in humans. An incubation period from exposure to clinical presentation is 1-21 days (Currie et al., 2000c), and depends on routes of infection. Routes of infection include cutaneous inoculation, inhalation, aspiration, or ingestion (White, 2003, Cheng and Currie, 2005, Limmathurotsakul and Peacock, 2011). An infection through inhalation is hypothesized to occur during extreme weather events (Currie et al., 2010).

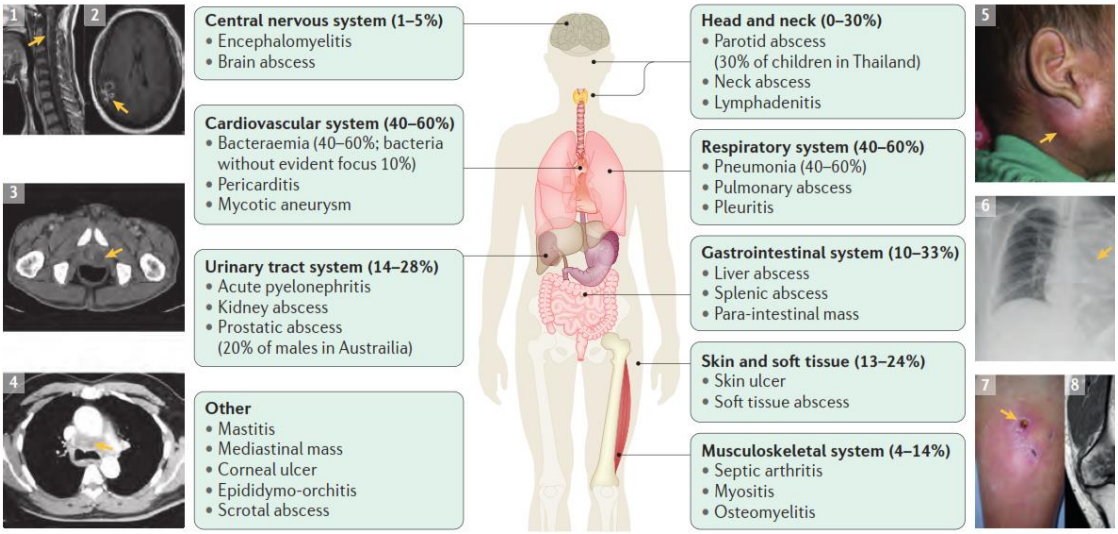
Patients with melioidosis can present with a wide range of clinical presentations, including an acute, chronic, latent and recurrent melioidosis. Acute and chronic infection are defined as duration of symptoms less than 2 weeks and more than 2 months prior to full clinical presentation, respectively (Currie et al., 2000a). About 80% of melioidosis patients presenting in endemic areas have an acute infection (Currie et al., 2000a, Currie et al., 2010). The proportion of patients with asymptomatic *B. pseudomallei* infection or latent melioidosis, in which the organism remains dormant in the host body for months or years, is unknown. Nonetheless, the proportion of patients presenting with an activation of latent

infection is not high. Recurrent melioidosis is defined as the development of signs and symptoms of melioidosis after a satisfactory initial treatment of the first infection (Sarovich et al., 2014). Recurrent melioidosis could be due to relapse of the original strain from the first infection or due to a re-infection of a new strain of *B. pseudomallei*.

Clinical manifestations of melioidosis include bacteraemia (55%), pneumonia (51%), chronic lung diseases (26-36%), genitourinary (14%), skin infection (13%), no evident focus (12%), neurological (3%), and osteomyelitis/septic arthritis (4%) (Currie et al., 2010, Limmathurotsakul et al., 2006a, Meumann et al., 2012, Currie et al., 2004). Localized infections, such as skin and soft tissue infection (38%) and parotitis (30%), are common presentations among children with melioidosis in Southeast Asia (Lumbiganon et al., 2011). Systemic infections with bacteraemia and pneumonia together account for up to 39% of all pediatric cases (Lumbiganon and Viengnondha, 1995, Turner et al., 2016).

There are a few differences in the clinical manifestations of melioidosis between Southeast Asia and Australia. First is that suppurative parotitis, which accounts for up to 40% of paediatric meloiodosis in Thailand and Cambodia (Lumbiganon and Viengnondha, 1995), is rare in Australia. Second, prostatic melioidosis and neurological melioidosis (especially brain stem encephalitis) present in 18% of male melioidosis patients, and 4% of all melioidosis patients in Australia (Currie et al., 2000b) but are less frequently observed in Thai patients. Figure 1-5 shows a summary of clinical manifestations of melioidosis by organ system.

Figure 1-5 Summary of clinical manifestation of melioidosis by Wiersinga et al (Wiersinga et al., 2018)



The case fatality rate (CFR) of melioidosis is about 14% in Australia and about 40% in Thailand (Currie et al., 2010, Limmathurotsakul et al., 2006a, Chierakul et al., 2005a). The CFR of patients with known co-morbidities in Australia ranges from 15% to 26% (Currie et al., 2004). Overall, co-morbidities were not associated with mortality except for chronic renal disease which increases the risk of mortality with a relative risk of 2.05 (95% confident interval 1.15-3.65) (Currie et al., 2004).

Patients who survive a primary episode of melioidosis may experience recurrent melioidosis in about 10%-17% of cases in Thailand (Chaowagul et al., 1993, Limmathurotsakul et al., 2008), 6% in Australia (Currie et al., 2010), 10% in India (Halim et al., 2017), and 2% in Lao PDR (Rachlin et al., 2016). *B. pseudomallei* is difficult to eradicated from the human body and this could lead to a relapse. The recurrent episode could be due to a relapse of the original infection or a new infection of a new strain of *B. pseudomallei*. Recurrence is classified as relapse when the isolates of the first and subsequent episodes of melioidosis are indistinguisable, as determined by genotyping (Limmathurotsakul et al., 2006b). Of all recurrent melioidosis, 65%-75% are dure to relapse

with the remaining proportion (25%-35%) associated with re-infection (new infections/ infected with different strains) (Limmathurotsakul et al., 2008, Sarovich et al., 2014). Clinical manifestations of recurrent melioidosis were similar to the primary episodes, including bacteraemia, pneumonia, liver and splenic abscesses, skin or soft tissue infection, and septic arthritis and osteomyelitis (Limmathurotsakul et al., 2006b). Bacteraemia and disseminated melioidosis occurrence during the primary infection is associated with an increased risk of relapse (Limmathurotsakul et al., 2006b, Currie et al., 2010).

The duration from the primary episode to relapse episode ranges from 2 to 28 months, while duration to re-infection ranges from 9 to 169 months (Sarovich et al., 2014, Currie et al., 2010, Limmathurotsakul et al., 2008). The duration from the primary episode to relapse observed in Thailand is shorter than Australia (median 9.4 months versus 24 months, respectively) (Limmathurotsakul et al., 2008, Sarovich et al., 2014). Relapse of melioidosis is associated with insufficient duration of initial intensive therapy, the duration of eradication treatment, and low patient-compliance to the eradication therapy (Currie et al., 2000a, Limmathurotsakul et al., 2006b, Sarovich et al., 2014, Chaowagul et al., 1993). In Thai patients, poor adherence to the eradication therapy is associated with increasing risk of re-presenting with clinical deterioration prior to completion of the 2 phases of therapy, and the relapse of melioidosis (Limmathurotsakul et al., 2006a, Wiersinga et al., 2018). In Darwin, Australia, lengthening intravenous parenteral therapy (ceftazidime and/or meropenem) to an average of 4-week was found to be effective, and the relapse rate declined from 6.4% to 1.2% from 1989 to 2012 (Sarovich et al., 2014). The CFR of patients with recurrent melioidosis is lower than that of the primary infection; 24% vs. 49% in Thailand,

respectively (Limmathurotsakul et al., 2006b) and 7% vs. 17.9% in Australia, respectively (Currie et al., 2010).

1.3.4 Diagnosis and identification of *B. pseudomallei* in clinical specimens

A laboratory diagnosis is necessary for the definite diagnosis of melioidosis, because clinical manifestations of melioidosis vary and can resemble a number of other infectious diseases. During the diagnostic process, melioidosis should be considered in febrile patients who are living in or have travelled from melioidosis endemic regions (Currie, 2015, White, 1994).

1.3.4.1 Bacterial culture

The gold standard diagnostic test for melioidosis is bacterial culture. Melioidosis is diagnosed if *B. pseudomallei* can be isolated from any clinical specimens. It has 100% specificity, but its sensitivity is as low as 60%. Sensitivity of culture largely depends on the number of clinical specimens collected for culture, volumes of blood specimen collected for culture, culture media used, and the experience of laboratory personnel in identifying *B. pseudomallei* (Limmathurotsakul et al., 2010a).

Blood culture should be taken on all suspected melioidosis patients because bacteraemia is a common clinical manifestation of melioidosis. Conventional culture methods and automated culture systems (e.g. BACTEC and BacT/ALERT) are widely used for blood culture (Hoffmaster et al., 2015, Teerawattanasook et al., 2014). Conventional culture is performed by incubating either home-made or commercial blood culture bottles, visually observing bottles regularly, and identifying bottles with cloudy broth. Then the cloudy broth is sub-cultured for bacterial identification. Automated culture system will automatically identify bottles with bacteria growth. For example, the BacT/Alert system (Organon

Teknika Corp., Durham, N.C.) uses colorimetric detection of CO_2 produced by growing microorganisms in the blood culture bottle.

Then, the isolated organism can be identified with either conventional methods or an automated bacterial identification method (e.g. Vitek2 and MALDI-TOF MS). The conventional method is largely based on Gram stain, colony morphology, oxidase test, biochemical tests, and antimicrobial susceptibility testing. Automated bacterial identification methods are based on either automated biochemical testing (e.g. Vitek2, Biomeriuex, USA) or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (i.e. MALDI-TOF MS). The accuracy of the identification made by automated systems is based on the strains database embedded in the system. Nonetheless, both conventional and automated identification methods share a major pitfall in that *B. pseudomallei* may be discarded as an environmental contaminant and no identification performed. In addition, misidentification of *B. pseudomallei* as *Pseudomomas* species or other *Burkholderia* species is also common with both conventional and automated identification methods, particularly if laboratory personnel are not aware of *B. pseudomallei* and a comprehensive database is not used, respectively (Podin et al., 2013).

Several strategies can be employed to increase accuracy of *B. pseudomallei* detection and identification. First, clinicians should collect all available clinical specimens, including blood, urine, sputum, pus, skin lesion swabs, throat swab and rectal swab for bacterial culture. Second, for non-sterile specimens, laboratory personnel should use a selective medium such as Ashdown broth, Ashdown agar or commercial *B. cepacia* media. These selective medium contain a mixture of crystal violet, colistin or polymyxin B and gentamicin that facilitate growth of *B. pseudomallei* over other organisms (Hoffmaster et al., 2015,

Peacock et al., 2005, Wuthiekanun et al., 1990). Third, laboratory personnel should identify and perform antimicrobial susceptibility testing for all Gram-negative oxidase-positive bacilli grown from all clinical specimens, particularly blood culture. *B. pseudomallei* is generally resistant to aminoglycodises (e.g. gentamicin), colistin, and polymyxin but susceptible to amoxicillin/ clavulanic acid (Podin et al., 2014). Finally, it is recommended not to discard any non-*Pseudomonas aeruginosa*, Gram-negative and oxidase-positive bacillus isolated from clinical specimens until definite identification of non-*B. pseudomallei* is made (Hoffmaster et al., 2015, Currie, 2015).

In addition, a few methods may be used in adjunct with conventional and automated bacterial identifications. These methods include a commercial API 20 NE kit (Dance et al., 1989b), *B. pseudomallei*-specific latext agglutination (Anuntagool et al., 2000a), and real-time polymerase chain reaction (PCR) targeting the *B. pseudomallei* type III secretion system (TSS1) gene cluster (Novak et al., 2006).

1.3.4.2 Serological diagnostic tests

Serological tests have limited utility in diagnosing melioidosis in endemic areas. The methods used to evaluate antibodies against *B. pseudomallei* antigens for diagnosis of melioidosis are the indirect hemagglutination assay (IHA) and enzyme-linked immunoabsorbent assays (ELISA).

The IHA detects the level of antibodies (IgG and IgM) against crude *B. pseudomallei* antigens, and is widely used worldwide. However, the method lacks antigen standardization, and its accuracy is low. IHA has specificity of around 60% (Chantratita et al., 2007b, Suttisunhakul et al., 2015, Suttisunhakul et al., 2016) and sensitivity ranged from 41% to 56% (Cheng et al., 2006). In areas endemic for melioidosis, the IHA should not be used for

diagnosing melioidosis. This is because populations in endemic regions have high background seropositivity due to the repeated exposure to *B. pseudomallei* in the environment (Harris et al., 2009, Cheng et al., 2006). Nevertheless, a laboratory study showed low levels of antibodies to *B. pseudomallei* in the serum of US donors (IHA titers ranged from <1:10 to 1:40), suggesting that IHA is useful for evaluation of exposure to *B. pseudomallei* (Suttisunhakul et al., 2016, Hoffmaster et al., 2015).

An ELISA method measures antibodies to specific antigens or targeted proteins of *B. pseudomallei*, e.g. O-polysaccharide (OPS) and hemolysin co-regulated protein 1 (Hcp1), respecitevely, which show potential diagnostic utility for melioidosis (Suttisunhakul et al., 2016, Pumpuang et al., 2017). An O-antigen based ELISA detecting IgG provided a better diagnostic accuracy with a sensitivity of 72% and specificity of 98% when compared to a capsular polysaccharide based ELISA and the IHA (Suttisunhakul et al., 2016). A recent study showed that an ELISA using a combination of *B. pseudomallei* specific OPS and Hcp1 (combined Hcp1/OPS-ELISA) performed better at detecting antibodies in the serum of melioidosis patients on admission day (Pumpuang et al., 2017). This could potentially be useful for diagnosing melioidosis; however, this test is still under the development and requires further evaluation.

1.3.4.3 Rapid diagnostic tests

Rapid diagnostic tests for melioidosis are crucial for improving the outcome of meliodosis. However, currently none of these rapid diagnostic and point-of-care tests for melioidosis are commercially available.

A lateral flow immunoassay (LFI) has been developed to detect capsular polysaccharide (CPS) specific to *B. pseudomallei*. A laboratory study showed that 98.7% of 77 strains of *B.*

pseudomallei react with the LFI assay, whereas 97.2% of near neighbor species were not reactive to the assay (Houghton et al., 2014). Therefore, the LFI could be used as a supplementary test for bacterial identification (Houghton et al., 2014).

An Active Melioidosis Detect™ lateral flow immunoassay (ADM LFI; InBios International, USA) has been evaluated with clinical specimens in Thailand, Lao PDR, India and Australia (Houghton et al., 2014). The test shows high specificity when compared with bacterial culture as the gold standard. Sensitivity of the test varies with type of specimen, ranging from 33% when tested with serum specimens (Wongsuvan et al., 2018), 33% when tested with sputum, 47% when tested with pus, 87% when tested with urine, and 99% when tested with turbid blood culture bottles (Woods et al., 2018).

The immunofluorescence assay (IFA) is a rapid diagnostic test that can be used to identify *B. pseudomallei* in clinical specimens directly and can be used to identify colonies of *B. pseudomallei* from bacterial culture. This IFA is used in a specialized laboratory in Thailand. The IFA identifies *B. pseudomallei* by using a monoclonal antibody against CPS and fluorescent dyes. The organism can then be observed using a fluorescence microscope (Tandhavanant et al., 2013, Chantratita et al., 2013). IFA has high specificity and can give a rapid result within 15 minutes. Nonetheless, the diagnostic sensitivity of the IFA is lower than bacterial culture (Wuthiekanun et al., 2005). A recent study shows high utility in detection of *B. pseudomallei* in turbid blood culture bottles and sterile fluid with sensitivity (99% and 100%, respectively) and specificity (100% in both specimens) (Woods et al., 2018). The IFA has lower utility in detection of the organism when tested with pus (sensitivity 66.7% and specificity 90.7%), and sputum (sensitivity 100% and specificity 85%) (Woods et al., 2018).

A rapid immunochromatography test using hemolysin-coregulated protein as a target antigen (Hcp1-ICT) has been developed recently as a point-of-care test for the serological diagnosis of melioidosis (Phokrai et al., 2018). The Hcp1-ICT detects IgG antibody specific to Hcp1. The Hcp1-ICT was evaluated on human serum samples obtained from four groups of participants, including (1) culture confirmed melioidosis patients, (2) healthy blood donors from Northeast Thailand, (3) healthy blood donors from the US, and (4) patients infected with other organisms from Northeast Thailand. The sensitivity of Hcp1-ICT was 88.3% in comparison with bacterial culture. The specitivities in Thai donors and US donors were 86% and 100%, respectively (Phokrai et al., 2018). This test is another potential point-of-care test of melioidosis.

In Thailand, the commonly used diagnostic tests for melioidosis are serological tests (IHA) and bacterial culture (conventional culture and automated bacterial culture system [BACTEC or BacT/ALERT]). The IHA is still commonly misused to diagnose melioidosis in Thailand, where the disease is highly endemic. Availability of microbiology laboratories is limited to secondary or tertiary hospitals, and they are absent in community hospitals. At community level, clinicians usually use the IHA for the diagnosis of melioidosis. A titer of antibody against *B. pseudomallei* of at least 1:160 is widely used as a cut-off for the diagnosis of melioidisis in Thailand, although it is proven to have low specificity. The latex agglutination assay and IFA are available in specialized laboratories belonging to research institutes in Thailand.

1.3.5 Treatment of melioidosis

Administration of antimicrobials effective against *B. pseudomallei* is crucial for improving the outcome of melioidosis (White, 1994, Wuthiekanun and Peacock, 2006,

Currie, 2015). In areas where melioidosis is endemic, immediate administration of specific antimicrobial against *B. pseudomallei* is recommended to all patients with suspected melioidosis (White, 1994).

In vitro studies show that *B. pseudomallei* is intrinsically resistant to penicillins, ampicillin, first- and second-generation cephalosporins, most of third-generation cephalosporins, gentamicin, aminoglycodises, and polymixin (Puthucheary and Parasakthi, 1987, Ashdown, 1988, Dance et al., 1989a, Yamamoto et al., 1990). There is a limited number of antimicrobials possessing bactericidal activities against *B. pseudomallei*. These include ceftazidime, imipenem, meropenem, piperacillin, and amoxicillin-clavulanate (Dance et al., 1989a, Yamamoto et al., 1990).

The treatment of melioidosis comprises of two phases; an initial intensive therapy with a parenteral antimicrobial for at least 10-14 days, followed by oral eradication therapy for 12-20 weeks (Lipsitz et al., 2012, Dance, 2014).

In 1986 the result of a large randomized control trial from Thailand led to a change in the first-line parenteral antimicrobial for melioidosis from ‘conventional therapy’ (a combination of chloramphenicol, doxycycline, trimethoprim, and sulphamethoxazole) to ceftazidime (White et al., 1989). The current drugs of choice for the initial treatment are ceftazidime or meropenem (White et al., 1989, White, 2003, Chierakul et al., 2005a, Currie, 2015). There are no clinical trials showing that treatment with meropenem has a lower mortality outcome than treatment with ceftazidime. Nonetheless, meropenem is recommended for severe melioidosis in Australia, due to its superior outcome observed in a non-trial study (Currie, 2014, Cheng et al., 2004).

The oral eradication treatment phase aims to prevent the recrudescence and relapse of melioidosis (White et al., 1989, White, 2003, Chierakul et al., 2005a). The recommended oral antimicrobial regimen is trimethoprim-sulfamethoxazole alone for 12-20 weeks (Chetchotisakd et al., 2013).

1.3.6 Prevention of melioidosis

Melioidosis is a preventable disease. In endemic areas, the recommended preventive measures include avoiding direct contact with soil and water, wearing protective gear (i.e. gloves and boots) when in direct contact with soil or water if direct contact with soil or environmental water is necessary, washing skin thoroughly after any exposure, and drinking only boiled or bottled water (Queensland Government., 2010, Suntornsut et al., 2016). However, only a small proportion of people follow such recommendations (Suntornsut et al., 2016). Public health interventions such as a behaviour change programme could be implemented by the MoPH. A feasibility study shows that a multifaceted prevention programme could lead to the adoption of recommended preventive behaviours. However, commitment and action by the government are essential for these preventive programmes to occur and be successful (Suntornsut et al., 2018).

Laboratory accidents involving exposure to *B. pseudomallei* can lead to infection, and good laboratory practices are required to prevent that (Peacock et al., 2008). The organism should be handled by trained personnel within a Biosafety Level 3 (BSL-3) facility (or national equivalent); laboratory practices specified by the respective national legislative and institutional biosafety committees should be used. However, diagnostic laboratories in resource-poor settings across tropical countries rarely have access to BSL-3 facilities; such laboratories can adapt many of the practices and work safely in a BSL-2 laboratory for little

or no extra cost. Safe laboratory practices will serve to minimize the risk of exposure to laboratory workers.

In general, laboratory workers should obtain organism- and site-specific training that includes orientation training for new workers and annual refresher training for all workers. Work should be conducted in a biologic safety cabinet and gloves should always be worn when manipulating clinical specimens suspected colonies of *B. pseudomallei* and *B. pseudomallei*. Respiratory protection must be used during centrifugation. Sealed cups should be used in all centrifuges, and these should be opened only in a biologic safety cabinet. In addition, research laboratories require clearly defined readiness guidelines and preparation in the event that one or more persons require postexposure prophylaxis (PEP) (Peacock et al., 2008).

1.4 Animal melioidosis

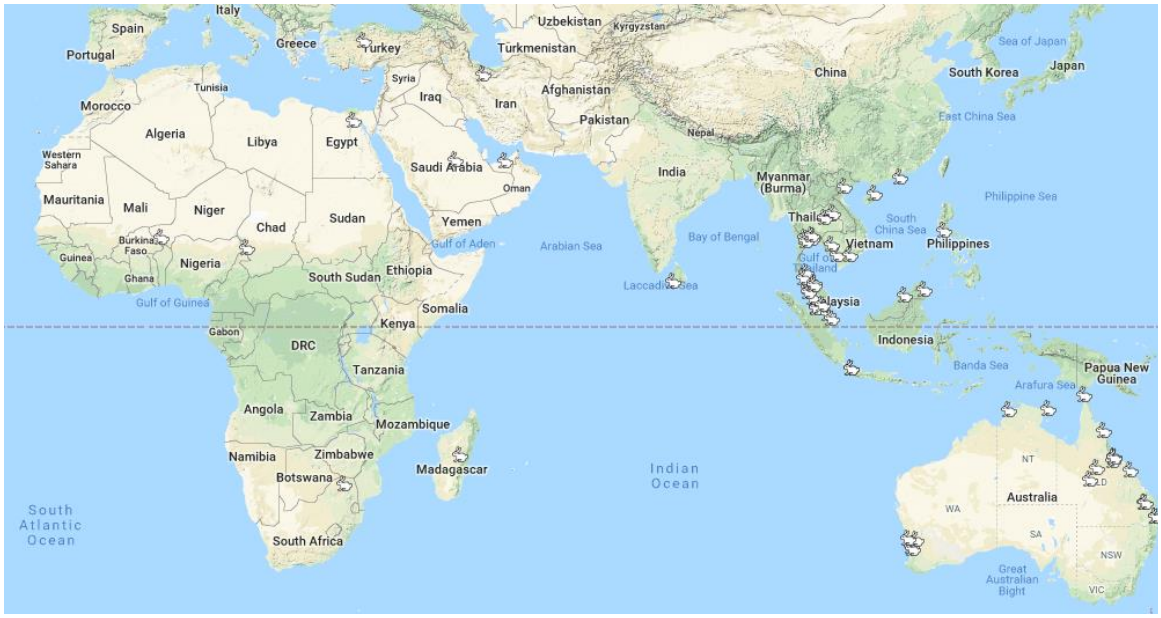
The epidemiology and global distribution of animal melioidosis is poorly understood. From 1917 to 2018, cases of animal melioidosis have been published from 23 countries worldwide (Figure 1-6). Most of the publications of animal melioidosis are from Australia, Malaysia, Indonesia, Thailand and Singapore (Choy et al., 2000, Limmathurotsakul et al., 2012a, Sim et al., 2018), all of which are countries endemic for human melioidosis (Golledge et al., 1992). Sporadic imported animal melioidosis cases have been reported in countries outside endemic areas; including the United States (Ryan et al., 2018, Butler et al., 1971), United Kingdom (Dance et al., 1992), and France (Mollaret, 1988). It is likely that melioiosis is also endemic in animals in countries where melioidosis is endemic in humans but animal melioidosis has never been reported.

A wide range of animal species is susceptible to *B. pseudomallei* infection, including pigs (Millan et al., 2007), poultry, sheep, lambs, goats, caprine (Thomas et al., 1988), cattle (also calves), buffalo calf, horse, alpaca (Lama pacos), boar, wallabies, koala, camels, orang-utan (Pongo pygmaeus), a macaque monkey (Macaca nemestrina), cats, birds, marine mammals (such as dolphin, whale, seals), and zoo animals (www.melioidosis.info). Animals are largely exposed via the environment, and routes of infection are possibly through ingestion, mucous membranes contamination and abraded skin inoculation (Rush and Thomas, 2012a).

Clinical manifestations in animal melioidosis range from acute infection with fever, septicaemia, pneumonia, gastroenteritis, diarrhea, infection in skin or parotid gland or mastitis to a chronic infection with abscesses in multiple organs (lung, liver, spleen) and arthritis (Tonpitak et al., 2014, Rush and Thomas, 2012a). *B. pseudomallei* can be isolated from multiple types of organs and specimens; including blood, pus, lung, liver, kidney, spleen, gonad, and skin (Sim et al., 2018, Limmathurotsakul et al., 2012a, Tonpitak et al., 2014). Diagnostic methods for animal melioidosis include isolation of *B. pseudomallei*, serological assays (IHA, ELISA, complement fixation testing [CFT], and IFA) and molecular methods (e.g. PCR). Isolation of the organism provides a definitive diagnosis of meloidosis. Serological assays such as IHA and CFT are used to evaluate infection status and exposure to the pathogen for surveillance purpose. However, serological assays cannot be used to provide a definite diagnosis of a current infection (Rush and Thomas, 2012a).

Figure 1-6 Distribution of animal meliodosis (www.melioidosis.info)

Image of rabbit represent location of published animal meliodosis.



In Thailand, animal melioidosis has been reported in a number of species. These include pigs, cattle, sheep, goat, crocodile, deer, horse, and zoo animals (for example chimpanzee, orangutan, kangaroo, zebra, camel, meerkat and cheetah) (Limmathurotsakul et al., 2012a, Tonpitak et al., 2014, Sommanustweechai et al., 2013, Kasantikul et al., 2015).

Animal melioidosis is one of the 20 diseases listed in the animal disease surveillance system conducted by the Bureau of Disease Control and Veterinary Services (BODCVS), Department of Livestock Development, Ministry of Agriculture and Cooperatives (MoAC), Thailand. However, the numbers of reported cases of animal melioidosis are low. Five years of animal surveillance data (2013 to March 2018) shows 14 cases of animal melioidosis; 13 in goats, and 1 in sheep (BODCVS, MoAC., 2015).

It is possible that a number of wild animals also acquire melioidosis but it is rarely reported. Routes of infection in animals in Thailand are unclear. It is hypothesized that

animals acquire *B. pseudomallei* through skin inoculation (Kasantikul et al., 2015). IHA is an official test for the diagnosis of animal melioidosis in the Thai animal surveillance system; seropositivity is defined as a titer ≥1:160 (BODCVS). Nonetheless, for a definite diagnostic of melioidosis in zoo animals, the methods should be bacterial culture or PCR assays (Kasantikul et al., 2015). A study in Thailand showed that culture-confirmed melioidosis in animals occurs in all six geographical regions in Thailand; including North, Northeast, Central, East, West and South, Thailand (Limmathurotsakul et al., 2012a).

1.5 Environmental *B. pseudomallei*

1.5.1 Distribution of environmental *B. pseudomallei*

The presence of environmental *B. pseudomallei* indicates a risk of acquiring melioidosis in both humans and animals living in those areas. However, the global distribution of this pathogen is still unclear. To date, environmental *B. pseudomallei* has been isolated from soil and/or water in East Asia (China, Hong Kong, Taiwan), the Middle East (Iran), Southeast Asia (Thailand, Cambodia, Laos, Viet Nam, Malaysia, Singapore), South Asia (India, Sri Lanka, Bangladesh), Australia (Australia, Papua New Guinea), Latin America and Caribbean (Brazil, Peru, Haiti), and Africa (Madagascar, Burkina Faso, Niger, Ivory Coast, Gabon) (www.melioidosis.info) (Figure 1-7).

Figure 1-7 Worldwide distribution of environmental *B. pseudomallei* (www.melioidosis.info)

The discovery of the organism in soil or water is often part of a follow-up investigation of a cases or cases of human or animal melioidosis (Wiersinga et al., 2015). Conversely, defining the distribution of the pathogen in the environment before melioidosis cases are reported can help raise awareness of the disease, increasing the chance of melioidosis identification, and leading to disease management and control (Limmathurotsakul et al., 2013a). A good example is from Lao PDR, where *B. pseudomallei* in soil was discovered in 1998, leading to identification of melioidosis cases in 1999 (Limmathurotsakul et al., 2013a). Environmental sampling (soil and/or water) is essential for determining the presence of the organism in the natural environment and creating a melioidosis risk map.

1.5.2 Soil sampling for the detection of *B. pseudomallei*

Obtaining a false negative for the presence of *B. pseudomallei* provides improper assurance of absence of risk to the population in the affected areas. Because *B. pseudomallei* unevenly distributes in the soil, poor study design and conduct of soil sampling may lead to

a false negative result (Limmathurotsakul et al., 2010c). For example, a study may collect an insufficient number of soil samples, collect insufficient sampling sites, collect low amounts of soil per sample, select sampling sites unsystematically, poorly handle samples, and poorly select culture techniques. Choices of study design and site selection should be based on the study objectives. Several sampling strategies should be considered during study design, including the total number of sampling sites, the total number of samples per site, distance between sampling points, sampling seasons, depth of soil sampling, amount (weight) of soil collected, conditions of sample transportation, and methods for isolation of *B. pseudomallei* (Limmathurotsakul et al., 2013a).

1.5.2.1 Sampling site selection, number of samples and distance between sampling points

A previous study using spatial statistics shows that soil samples taken from sampling points adjacent to sampling points culture positive for the organism are more likely to yield a positive culture (Limmathurotsakul et al., 2010c). Likewise, soil samples taken from sampling points adjacent to sampling points culture negative for the organism are also more likely to yield a negative culture (Limmathurotsakul et al., 2010c). To lower the risk of false negative results, it is recommended to use a sample size calculation to derive the total number of sites. In Thailand, it is recommended to collect at least 100 sampling points per site, with a distance between sampling points of 2.5 to 3.5 meters (Limmathurotsakul et al., 2010c). The strategy is in line with the consensus guideline for soil sampling developed by the "Detection of Environmental *Burkholderia Pseudoamallei* Working Party (DEBWorP)" (Limmathurotsakul et al., 2013a) (Table 1-1).

Table 1-1 Published and recommended soil sampling strategies and recommended methodologies of isolation of *B. pseudomallei* from Soil by DEBWorP (Limmathurotsakul et al., 2013a)

Sampling strategy	Published sampling strategy	Consensus guideline
Sample size calculation	Not stated and often low sample size	Sample size calculation should be presented and should correspond with the aims of the study
Sampling site selection	Variable, including random site selection and practical considerations (e.g. sampling at points along a main road)	For pilot studies that are conducted to identify environmental *B. pseudomallei* in areas where sampling has not been done previously, choose sites most likely to be positive based on available information such as areas around households or working fields of melioidosis patients. If such information is unavailable, use the GIS program to randomly select sampling sites
		For large environmental surveys in areas where *B. pseudomallei* is known to be present in the environment, use the GIS program to randomly select sites across the designated region
Sampling points per site	Ranged from 2 to 100 points per field	Use a fixed interval sampling grid
		To determine presence of *B. pseudomallei* in one field (around 50×50 sq meters), 100 points per site
		To determine presence or distribution of *B. pseudomallei* in a wider area, number of points per site and number of sites should be calculated based on geo-statistical sample size calculation which should provide the confidence level required
Distance between sampling point within a sampling site	1 to 5 meters, or not reported	If no prior information available for *B. pseudomallei* distribution in test area, take samples at a distance of 2.5 to 5 meters apart
		If prior information is available, samples should be taken at an optimal distance based on geo-statistical sample size calculation
Soil sampling depth	Ranged from 0 to 90 cm of depth	30 cm depth
Weight of soil sample per sampling point	Ranged from 2 to 1,000 gram of soil	10 gram of soil (put into universal tube)
Temperature during transportation of sample to laboratory	Variable, including room temperature and refrigerated temperature	At ambient temperature and away from direct sunlight or heat source
		Process soil samples as soon as possible

1.5.2.2 Seasons of soil sampling

The presence of *B. pseudomallei* in the soil during the wet season is speculated to be higher than during the dry season. Therefore, the probability of detecting the pathogen by soil sampling during the wet season may also be higher than during the dry season. An observational study observed that positivity for *B. pseudomallei* in the soil in wet season was higher than during the dry season (Thanapat et al., 2013). However, this study was conducted with a small number of soil samples per sampling site (4 samples per site), which may limit the generalisability of this result. A recent review shows that the difference in positivity rate for *B. pseudomallei* between two sampling seasons is still uncertain (Limmathurotsakul et al., 2013a). This implies that positivity rates are also associated with

other contributing factors (e.g. land usages and presence of animals) which can change over the time and the locations of sampling. Hence, it is important to take study objectives and all available information about the sampling areas into account when shaping sampling strategies.

1.5.2.3 Soil sampling depth and condition of sample transportation

A depth of 30 cm from the soil surface is the depth for soil sampling recommended by DEBWorP (Limmathurotsakul et al., 2013a). This choice of sampling depth is based on published evidence that the proportion of samples that are culture positive for *B. pseudomallei* is higher at 30 cm than at the surface (Thomas et al., 1979), but comparable to samples taken deeper than 30 cm (Knappik et al., 2015)**.**

A laboratory study shows that environmental *B. pseudomallei* survives optimally at a temperature between 24℃ and 32℃, and that the organism is killed by ultraviolet rays at 465 μW/cm^2 (Tong et al., 1996). Therefore, soil samples should be stored in a container at this optimal temperature range and protected from direct sun light or heat during transportation.

1.5.2.4 Extraction, detection, and isolation methods of *B. pseudomallei* from soil

A summary of a comprehensive review and details of the consensus culture guidelines is reproduced in Table 1-2. Methods used for differentiation of environmental *B. pseudomallei* are similar to those used for isolates obtained from clinical specimens.

Any colony suggestive of *B. pseudomallei* can be initially differentiated by Gram stain, oxidase test, arabinose assimilation test, and antimicrobial drug susceptibility testing (resistant to gentamicin and colistin but sensitive to amoxicillin-clavulanic acid). Then the organisms should be tested by a few confirmatory tests including latex agglutination, PCR

assay and API20NE (Limmathurotsakul et al., 2013a). Multilocus sequence typing (MLST) can be used as a confirmatory test and as a tool to determine genetics relatedness between isolates from different geographical regions reported in the public database.

Laboratory studies and one environmental study show that PCR-based methods (targeting TSS1) have a higher sensitivity to detect *B. pseudomallei* in the soil than bacterial culture (Brook et al., 1997, Trung et al., 2011, Kaestli et al., 2007, Knappik et al., 2015, Gohler et al., 2017). However, PCR-based methods alone will not be able to obtain bacterial isolates for confirmation and genetic studies. Therefore, PCR-based methods can be used together with culture, if available. Also, large studies to properly evaluate the sensitivity and specificity of PCR-based methods in the real environmental settings are still required.

Table 1-2 Published and recommended methodologies for the isolation of *B. pseudomallei* from Soil by DEBWorP (Limmathurotsakul et al., 2013a).

Methodologies	Published methods	Consensus guideline
B. pseudomallei extraction solution	Distilled water, normal saline, detergents or enrichment media	Threonine-basal salt plus colistin 50 mg/L (TBSS-C50 broth)
		Ashdown broth containing colistin and crystal violet is an alternative
Ratio of soil and extraction solution (wt/wt)	Ranged from 2:1 to 1:10	1:1 (10 gram of soil to 10 ml of TBSS-C50 or Ashdown broth)
Extraction method	Manual shaking, vortexing or orbital shaker	Vortexing for 30 seconds
		Manual mixing of soil is an alternative option, and may be required if sample is compacted
Techniques for detection of *B. pseudomallei*	Culture, PCR or animal inoculation	Culture (PCR could be added as an additional technique if available)
Protocol for culture	Variable, including direct culture on solid media and quantitation, and qualitative methods relying on broth enrichment	Incubate the specimen (universal tube with 10 gram of soil plus 10 ml TBSS-C50 or Ashdown broth) for 48 hours
Temperature of incubator	Variable, ranged from 37 to 42°C	40°C is recommended, and 37–42°C is an alternative option
Protocol for sub-culture	Variable	Subculture 10 μL of supernatant onto an Ashdown agar plate, and streak to achieve single colonies
		Incubate plate and examine every 24 hours for 7 days
Identification of *B. pseudomallei*	Variable, including basic microbiological tests (which include typical colony morphology, Gram stain, positive oxidase test, inability to assimilate arabinose, resistance to gentamicin and colistin with susceptibility to co-amoxiclav) and biochemical kits (including API20NE [105] and Vitek) with or without additional confirmatory tests (specific latex agglutination test [89], or a specific PCR assay [62,75,91,93])	Basic microbiological tests (which include typical colony morphology, Gram stain, positive oxidase test, inability to assimilate arabinose, resistance to gentamicin and colistin with susceptibility to co-amoxiclav) is mandatory plus at least one confirmatory test (API20NE, Vitek system, specific latex agglutination test [89] or a specific PCR assay [62,75,91,93], unless latex test or PCR assay was used during screening)
		Specific latex agglutination test [89], or a specific PCR assay [62,75,91,93] can be used a screening test

1.5.3 Ecological factors associated with environmental *B. pseudomallei*

B. pseudomallei is a saprophytic beta-proteobacteria, a facultative aerobe that uses respiration to harvest energy through oxidization of organic compounds, utilizing oxygen and nitrate in aerobic and anaerobic condition, respectively (Ian L. Pepper, 2009). Knowledge of environmental factors associated with the presence of the organism in the natural setting is limited and conflicting. Notable findings from both laboratory studies and environmental studies are summarized below.

1.5.3.1 Soil texture and water content/ moisture level

The persistence and survival of *B. pseudomallei* is influenced by soil type and by the level of soil moisture (Kaestli et al., 2015, Palasatien et al., 2008). This is because soil type is associated with the ability to hold water, oxygen, and nutrients which are essentials to the survival of soil microorganisms. For example, clayey soil holds water better than sandy soil (Ian L. Pepper, 2009). Soil studies have shown that the presence of *B. pseudomallei* is associated with clayey soil, due to its superior ability to hold water (thus higher moisture level) and support growth of the organism (Thomas et al., 1979). Findings from both laboratory studies and environmental surveys support this suggestion. Laboratory studies show that soil with water content less than 15% inhibits growth of the organism by 60 days (Chen et al., 2003) and soil with water less than 10% can lead to death of the bacterium by 70 days (Tong et al., 1996). Experimental studies show that adding water to the soil yields 3 times higher odds of detecting *B. pseudomallei* compared to control soil (Kaestli et al., 2015), and that an environmental *B. pseudomallei* strain can survive in intermittently irrigated soil for at least 113 days (after which the experiment was terminated) while the strain survived only 91 days in desiccated soil (Larsen et al., 2013). A number of

environmental studies in Australia (Kaestli et al., 2009, Larsen et al., 2013), in Thailand (Palasatien et al., 2008), and Gabon in Africa (Wiersinga et al., 2015) also found that soil samples culture positive for *B. pseudomallei* had higher water content or moisture than the soil samples negative for the organism.

However, *B. pseudomallei* can also be isolated from sandy soils (Smith et al., 1995, Palasatien et al., 2008, Baker et al., 2015, Larsen et al., 2013), which naturally contain lower water content and moisture level. *B. pseudomallei* can also be isolated from soil in harsh and desert-like environments in Central Australia (Yip et al., 2015). Laboratory experiments also confirm that *B. pseudomallei* can survive in desiccated environments (soil with 0% water content) for 30 days (Tong et al., 1996).

1.5.3.2 pH and acidic environment

An acidic environment is reported to be a preferable environment for *B. pseudomallei*. The organism can grow in an experimental broth at pH 4.5, in which *B. cepacia* and *B. aeruginosa* can not survive (Dejsirilert et al., 1991). In a number of laboratory studies, pH 4-8 is an optimal range for *B. pseudomallei* growth and survival (Tong et al., 1996, Wang-Ngarm et al., 2014, Chen et al., 2003, Kaestli et al., 2015). A laboratory experiment done in Thailand observed that growth of *B. pseudomallei* declines at pH 8, while the organism grows well at pH 4-7 (Wang-Ngarm et al., 2014). In another two studies, pH 9 was inhibitory to the survival of *B. pseudomallei* in normal saline after 26 days (Tong et al., 1996) and in soil after 2 days (Wang-Ngarm et al., 2014). In an environmental study, *B. pseudomallei* wass isolated from soil with a low pH ranging from 4 to 6 (Ngamsang et al., 2015, Palasatien et al., 2008). In another environmental study in Australia, the pH of soil samples positive for *B. pseudomallei* had a lower pH than the soil samples negative for the organism (range

of pH 5.2-5.8 vs. 6-7, respectively) (Kaestli et al., 2009). Multiple soil surveys from Thailand reported no difference in pH between soil positive and negative for *B. pseudomallei* (Sermswan et al., 2015, Thanapat et al., 2013, Ngamsang et al., 2015). For example, a soil survey observed pH range of 4.77-7.65 versus 5.35-7.7 in soil positive and negative for the organism, respectively (Sermswan et al., 2015).

1.5.3.3 Salinity and osmotic stress

B. pseudomallei grows well in a soil microcosm with a concentration of sodium chloride between 0% and 0.7% (Wang-Ngarm et al., 2014). As salinity increases up to >2.5%, growth of the organism decreases to below detectable level (Wang-Ngarm et al., 2014, Inglis and Sagripanti, 2006). The possible explanation for this is that the high salinity of 2.5% imposes physiological stress to *B. pseudomallei*. As a result, the organism goes into a viable but non-culturable state (VBNC) (Inglis and Sagripanti, 2006).

The salinity of natural soil is determined by measuring the electrical conductivity level (EC; ds/m). EC reflects the concentration of soluble salts in soil, including sodium, choline, magnesium, calcium, potassium and nitrate. A previous soil survey from Thailand did not find a difference of salinity level between soil culture positive and negative for *B. pseudomallei* (EC 0.03-0.93 vs. 0.03-0.17 ds/m, respectively) (Thanapat et al., 2013). An experimental study from Australia observed that the concentration of *B. pseudomallei* detected is negatively associated with the level of EC in the soil samples, and that garden soil with EC of 0.7 ds/m inhibits persistence of *B. pseudomallei* beyond 4 weeks after inoculation (Kaestli et al., 2015).

1.5.3.4 Soil nutrients

Nitrogen is an essential nutrient for microorganisms and plants (Ian L. Pepper, 2009). *B. pseudomallei* has an ability to change nitrate to nitrites during anaerobic respiration. Soils with presence of *B. pseudomallei* have significantly higher levels of nitrogen compared to soils negative for the organism (Palasatien et al., 2008). An experimental study shows that soils treated with high nitrate fertilizer enhance growth of the organism (Kaestli et al., 2015).

Iron is also important for microbial metabolism. In an experimental study, soils treated with iron ranging from 75 to 150 mg/kg improve growth of *B. pseudomallei* (Wang-Ngarm et al., 2014). This is consistent with a finding from an environmental survey which shows that soils positive for *B. pseudomallei* have higher levels of iron than negative soil; 45.4±18.4 vs. 17.9±7.2 mg/kg, respectively (Ngamsang et al., 2015). However, an Australian soil survey shows that sampled sites with high prevalence of *B. pseudomallei* have lower levels of iron than sampled sites with low prevalence of *B. pseudomallei;* 8-12 mg/kg vs. 160-180 mg/kg, respectively (Baker et al., 2015). Both environmental studies were restricted to two sampling sites. In an environmental study carried out in Thailand, soil samples which were culture positive for *B. pseudomallei* had a wide range of iron content from as low as 6.17 mg/kg to as high as 288.48 mg/kg (Thanapat et al., 2013).

Apart from nitrogen and iron, total organic carbon was found positively related to presence of *B. pseudomallei* (Palasatien et al., 2008, Wang-Ngarm et al., 2014). However, soils with low total carbon and high prevalence of *B. pseudomallei* were also observed in a soil survey in Australia (Baker et al., 2015).

1.5.4 Environmental *B. pseudomallei* in Thailand

In Thailand, little is known about the distribution of *B. pseudomallei* (Figure 1-8). Most of the environmental studies for *B. pseudomallei* have been limited to Khon Kaen (Palasatien et al., 2008) and Ubon Ratchathani provinces (Wuthiekanun et al., 1995a, Smith et al., 1995).

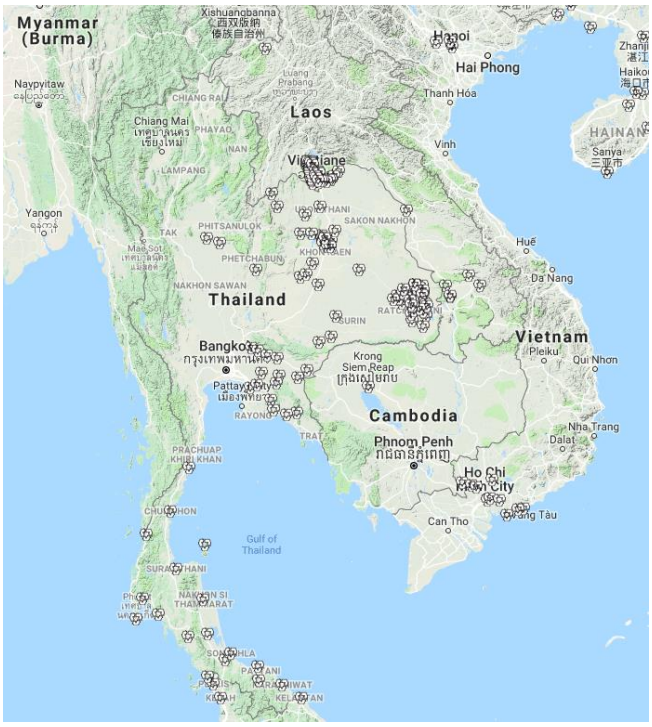
The very first large environmental survey to identify *B. pseudomallei* in Thailand was conducted between 1964-1967 (Finkelstein et al., 2000). Soils or water culture positive for the organism were found in Northeastern and the Southern regions (Finkelstein et al., 2000). A recent large soil survey was conducted in 1999. The highest *B. pseudomallei* positivity rates were reported in the Northeast (50.1%), followed by the Central (24.5%), the South (18.4%), and the North (13.8%) (Vuddhakul et al., 1999). Although soil culture positive for *B. pseudomallei* in the Central region was second after the Northeast, clinical cases had been rarely reported in the Central region. In fact, the majority of *B. pseudomallei* reported in this study in the Central region assimilated arabinose, suggesting that they were actually non-pathogenic *B. thailandensis* (Brett et al., 1998). In addition, this study was not conducted in the East region, where human melioidosis is reported. The sampling areas were limited to roadsides along the highway (Vuddhakul et al., 1999), which may not represent the risk areas where the rice farming population is normally exposed to the organism. Considering the incidence of melioidosis in the East region (Bhengsri et al., 2011a), it is predicted that *B. pseudomallei* is present in the soil in that area. A pilot study conducted by Vanaporn Wuthiekanun found that *B. pseudomallei* is occasionally found in the East region but less common than in the Northeast (personal communication, unpublished data).

To understand the factors associated with the presence of *B. pseudomallei* in rice fields, surveys for organism positivity and evaluation of soil characteristics should be carried out simultaneously. In Thailand, the sole analytical study of this type was performed in Khon Kaen province, northeast Thailand (Palasatien et al., 2008). The investigators reported that soil physiochemical properties associated with presence of the organism were pH 5-6, a moisture content >10%, higher chemical oxygen demand (COD) and higher nitrogen concentration (Palasatien et al., 2008). Nonetheless, this study cannot explain the presence or absence of *B. pseudomallei* in different regions outside northeast Thailand. Understanding the factors associated with presence of *B. pseudomallei* may provide useful information on how to eradicate or reduce the high concentration of *B. pseudomallei* present in the soil in Northeast Thailand.

The presence of other *Burkholderia* species may also be positively or negatively associated with the presence of *B. pseudomallei*. The non-pathogenic Gram-negative bacterium *B. thailandensis* has colony morphologies and antigenicities similar to those of *B. pseudomallei*, except that *B. thailandensis* can assimilate L(+) arabinose and *B. thailandensis* lacks the *B. pseudomallei*-like CPS. Other *Burkholderia* spp. that have been reported from other countries include *B. vietnamensis*, *B. ubonensis* and *B. oklahomensis* (Wiersinga et al., 2012). Nikolakakis et al. recently showed that *Burkholderia* species contained functional contact-dependent growth inhibition (CDI) systems, which may confer a competitive advantage on these bacteria over other species (Nikolakakis et al., 2012). An experimental study found that there is mutual inhibition between *B. pseudomallei* and *B. thailandensis* in culture medium (Ngamdee et al., 2015). Nonetheless, there is currently a lack of evidence supporting this finding in the natural environment. Therefore, the presence

of other *Burkholderia* species should be evaluated in the environmental survey as one factor potentially associated with the presence of *B. pseudomallei*.

Figure 1-8 Distribution of environmental *B. pseudomallei* in Thailand (www.melioidosis.info)



1.6 Surveillance and global burden of melioidosis

Infectious disease surveillance plays a crucial role for public health, as it can be used to guide resource allocation for diseases prevention, control and treatment programs developed by health policy makers. A disease surveillance system can also be used to monitor changes in disease frequency and in levels of risk factors. A disease surveillance system usually requires three integrated systems: data collection (e.g. case reports); data analysis; and timely information dissemination to guide interventions and policies (M'ikanatha et al., 2013, WHO, 2018). The main indicators for burden of disease are morbidity and mortality.

In addition, surveillance can also be used to assess changes in levels of environmental risk factors for the diseases. Such monitoring may forewarn of possible rises in rates of diseases associated with that environmental agent. Thus, surveillance for changes in either disease rates or levels of environment risk factors inform possible directions for reducing health threats in the future.

Active surveillance systems intensively determine the incidence and epidemiological characteristics of specific conditions within defined areas, and require dedicated extensive resources. Therefore, this type of surveillance is rarely applied in either resource-limited or developed countries. National surveillance system in most countries is based on passive surveillance systems, in which patients diagnosed with notifiable diseases are reported by the healthcare provider or by district health authorities (Gordis, 2013, M'ikanatha et al., 2013).

Melioidosis is not listed as a notifiable disease in many endemic countries, including Malaysia, Vietnam, Cambodia, Lao PDR, India, Sri Lanka, and the Phillipines. However, melioidosis is listed in the national surveillance systems in Singapore, Taiwan, Japan, Australia (Queensland, the Northern Territory and Western Australia) and Thailand. Melioidosis is not listed as a notifiable disease in the US; nevertheless, the US Centers for Disease Control and Prevention (CDC) has an additional surveillance system for agents considered to have bioterrorism potential and has classified *B. pseudomallei* as a Tier 1 select agent (agents that present the greatest risk of deliberate misuse with significant potential for mass casualties and pose a severe threat to public health and safety (CDC)). Figure 1-9 illustrates melioidosis cases reported in scientific publications according to a previous review using data from 1917 to 2013 (Limmathurotsakul et al., 2016).

Figure 1-9 Distribution of human melioidosis, which were reported in scientific publiactions from 1917 to 2013 (www.melioidosis.info)

Using mathematic modelling, Limmathurotsakul et al. predicted 165,000 melioidosis cases worldwide (Limmathurotsakul et al., 2016). However, only about 1,300 melioidosis cases were reported per year worldwide since 2010 (Limmathurotsakul et al., 2016). Underreporting of melioidosis may be associated with low awareness of the disease, lack of capacity or expertise in identifying the causative pathogen, and a lack of formal or inefficient reporting or surveillance system.

Awareness of melioidosis plays a critical role in disease recognition at all levels. Because awareness of the disease is usually very low among patients and clinicians, melioidosis is often not included as a possible cause of illnesses. Consequently, clinical specimens may not be collected for culture, and laboratory technicians may not be notified that an isolate should be suspected of being *B. pseudomallei*. This reduces the chance of detecting the pathogen tremendously, particularly in samples with mixed culture. As a result, melioidosis patients are rarely diagnosed – as depicted in the surveillance iceberg below

(Figure 1-10). Although awareness of melioidosis is relatively higher in well-known melioidosis-endemic areas (e.g. Thailand), reported cases are also believed to be just the tip of the iceberg and not representative of the true incidence of melioidosis (Dance, 1991).

Figure 1-10 Surveillance iceberg (Ketheesan , N. editor 2012)

Underreporting of melioidosis from all levels is a major contributing factor to unknown burden of melioidosis. As a result, melioidosis is not set as a priority disease that requires resource allocation for enhancing diagnosis, treatment, prevention and surveillance. This is happening in most, if not all, developing countries where melioidosis is known to be highly endemic.

1.7 Surveillance and burden of melioidosis in Thailand

1.7.1 National surveillance system in Thailand

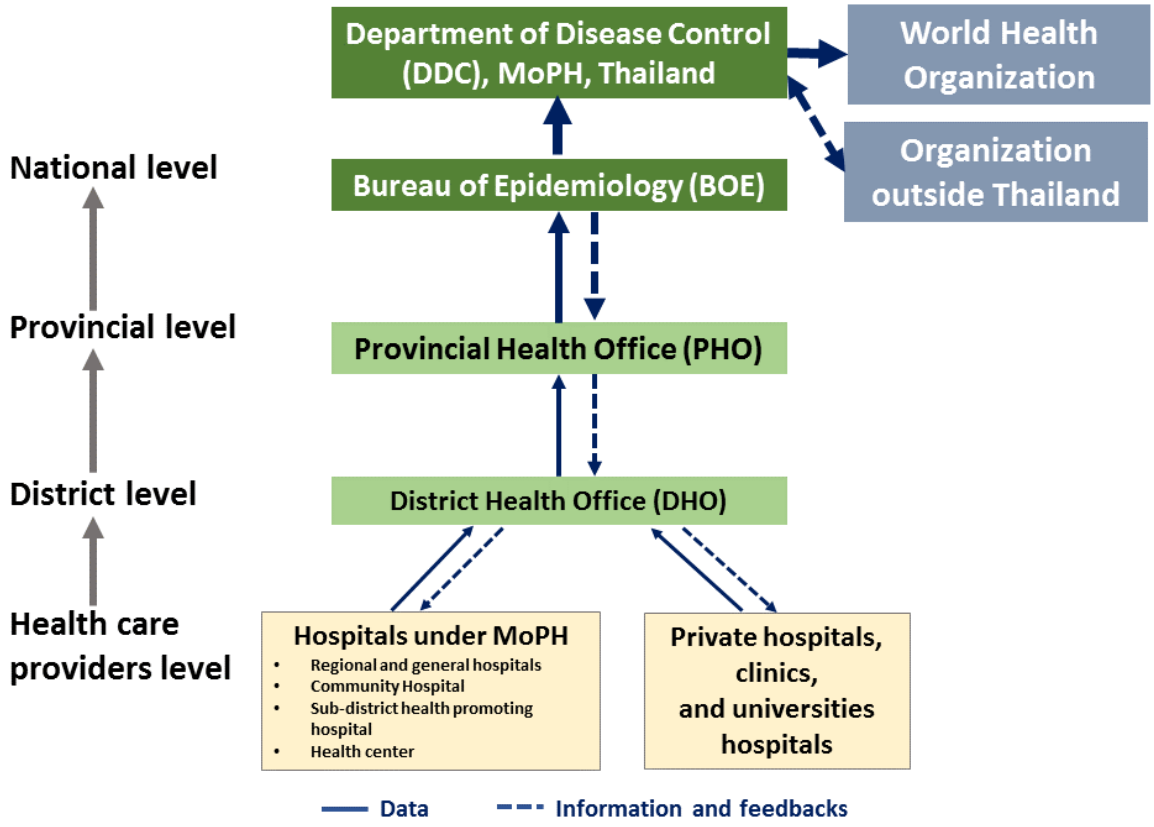
In Thailand, communicable diseases legislation was established in 1934 and has been regularly updated since, and the National Communicable Disease Surveillance system was established in 1968. The first Communicable Disease Surveillance system covered 14 infectious diseases, and this has been expanded since to cover 81 diseases including, since 2002, melioidosis.

The system is managed by the Bureau of Epidemiology (BOE), Ministry of Public Health (MoPH), Thailand. The list of notifiable diseases, case definitions, report forms, and the data system are defined by the BOE, MoPH. The flow of national surveillance data is depicted in Figure 1-11. In brief, first, local healthcare providers such as community hospitals, general and regional hospitals under MoPH, and hospitals under other ministry send case reports (a report form called “Report 506”) to the district health office (DHO). The DHO then sends the data to the provincial public health office (PPHO) (Figure 1-11). Then, PPHO retrieves and validates the data from hospitals under its jurisdiction, and sends the validated reports to the main database located in the BOE. Data from hospitals located in 77 provinces nationwide are analyzed, and an annual epidemiological report of all notifiable diseases is written by the BOE and submited to the Department of Diseases Control (DDC), MoPH. Policy makers regularly use the annual epidemiological reports as a main reference for resource allocation for disease diagnosis, treatment, prevention and control programs.

Prior to 2016, the notifiable disease system in Thailand was not mandatory. Since June 2016, reporting of culture-confirmed melioidosis cases from every hospital with a

microbiology laboratory, together with final outcome, has been mandated under the Communicable Diseases Act B.E. 2558 (A.D. 2015). Now, hospitals nationwide need to report all probable and confirmed cases of melioidosis. The BOE defined probable and confirmed cases of melioidosis as clinically compatible illness with laboratory diagnostics results. Probable clinical presentations of melioidosis include fever, septicaemia, single or multiple abscesses including cutaneous, splenic or hepatic abscess, septic arthritis, osteomyelitis, and abnormality of chest x-ray. Laboratory diagnosis criteria are IHA titer ≥1:160 from a single blood sample, or four-fold rising of two blood samples, or immunofluorescence antibody test (IFA) >1:400, or culture positive for *B. pseudomallei*. Reporting of melioidosis is required for all probable cases (e.g. cases with clinical symptoms and IHA ≥1:160). Confirmed melioidosis is defined as having clinical symtoms (mentioned above) and one or more laboratory diagnostic result: IHA titer ≥1:160 from a single blood sample, or four-fold rising of two blood samples, immunofluorescence antibody test (IFA) >1:400, or culture positive for *B. pseudomallei* from any specimens.

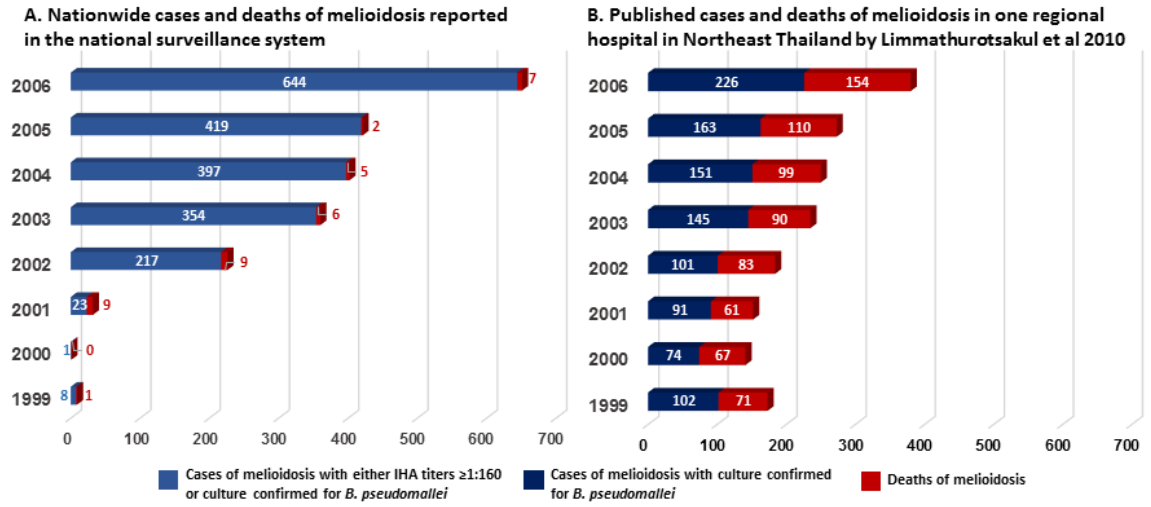
Figure 1-11 Flow of national surveillance data (Bureau of Epidemiology, 2016)



Records of Report 506 showed that a total of 39 patients died of melioidosis countrywide between 1999 and 2006. However, a retrospective study showed that there were more than 735 deaths from melioidosis in one regional hospital during the same period (Limmathurotsakul et al., 2010b) (Figure 1-12). These discrepancies in the numbers of deaths due to meliodosis suggest that the incidence and mortality of melioidosis recorded via the Report 506 system is substantially under-reported.

The BOE, MoPH, Thailand regularly publishes the summary data of notifiable diseases from Report 506 in the Annual Epidemiological Surveillance Report of Thailand. As that information is regularly used by policy makers, the burden of melioidosis is currently neglected. Therefore, the under-reporting of the incidence and mortality of melioidosis via the Report 506 system needs to be rectified.

Figure 1-12 Discrepancies between deaths of melioidosis in 1999-2006 reported in national surveillance system and published data



1.7.2 Burden of melioidosis in Thailand

In 1955, the first culture-confirmed melioidosis was reported from a province located in Central Thailand by Chittivej et al (Chittivej et al., 1995). Up until 1976, a total of 16 culture-confirmed melioidosis cases had been identified in Bangkok. As a result, melioidosis was raised as a threat to public health nationwide (Chayasirisobhon, 1976). With a rising of disease awareness among infectious disease physicians, reports of meloidosis cases have continued to increase. Up until 1985, over 700 culture-confirmed melioiodisis cases had been reported from many provinces in the Northeast (n=585), the North (n=61), the Central (n=102), and the South (n=6) (Punyagupta, 1989). It became clear that the Northeast is the most highly endemic region for melioidosis in Thailand.

Despite the increase in case reporting, improvements in diagnosis, and including melioidosis as a notifiable diseases in Thailand, the true burden of melioidosis in the country and in each region is still largely unknown. Thailand is divided into six geographical regions by the National Geographic Committee in 1977, including Northeast, North, East, West,

South, and Central Thailand (Kashino, 2014). Evidence of melioidosis in each geographical region is summarized below.

1.7.2.1 Northeast Thailand

Northeast Thailand is recognized as the most highly endemic region for melioidosis worldwide. Northeast Thailand is the largest region in Thailand by area (168,854 km^2) and consists of 20 provinces (The Bureau of registration Administration, 2013). Most of the published melioidosis cases in Thailand are from four provinces: Khon Kaen, Nakhon Panom, Udon Thani, and Ubon Ratchathani. These four provinces are where melioidosis research institutes are located.

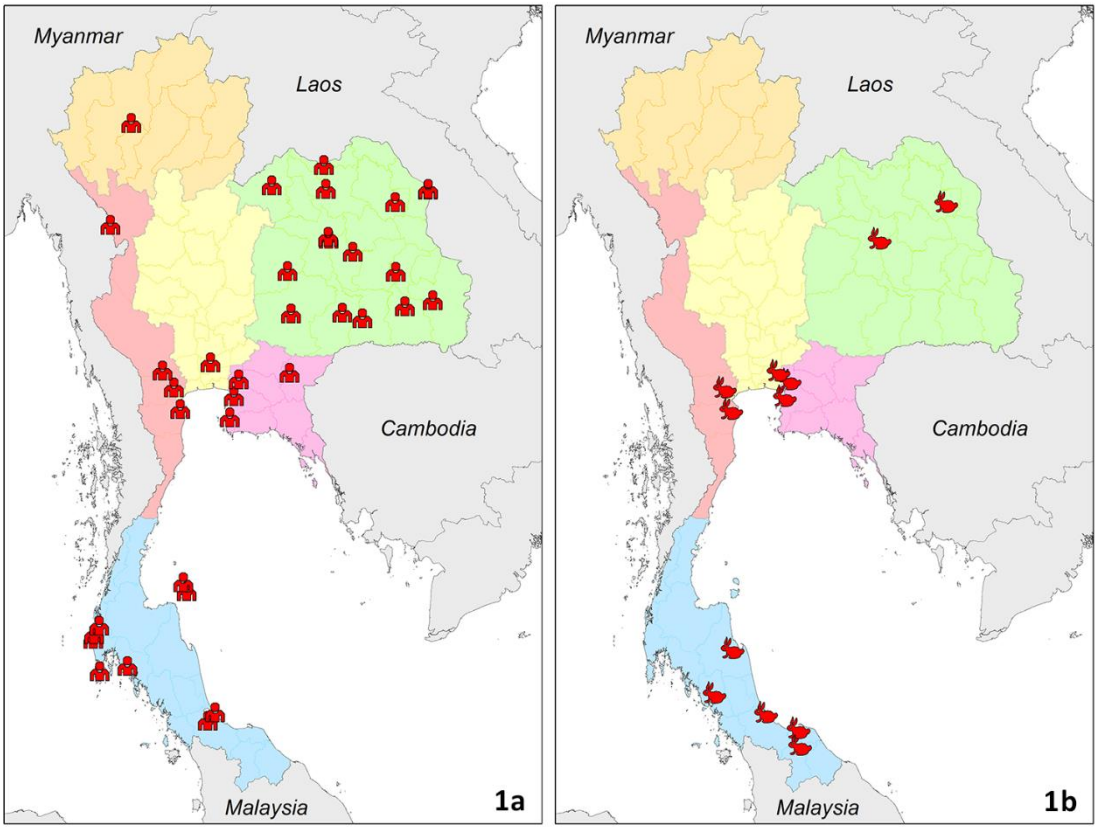
In 1997, 1,050 culture-confirmed melioidosis were observed in general and regional hospitals in Northeast Thailand; however, details of provinces and outcomes of those cases were not specified (Vuddhakul et al., 1999). From 1997 to 2006, a study reported that the incidences of culture-confirmed melioidosis cases observed at Sunpasitthiprasong Hospital, Ubon Ratchathani ranged from 141 to 380 cases per year (Limmathurotsakul et al., 2010b). Observed incidence rates of melioidosis in Ubon Ratchathani had substantially increased from 8.0 to 21.3 per 100,000 population per year between 2000 and 2006 (Limmathurotsakul et al., 2010b), and melioidosis is the third most common cause of death from infectious diseases in Northeast Thailand after HIV/AIDS and tuberculosis (Limmathurotsakul et al., 2010b).

In a study from Nakhon Panom hospital, the total number of culture-confirmed melioidosis cases sharply rose from 130 cases in 2003-2005 to 330 cases in 2006-2008 because an automated bacterial culture system was fully implemented in 2006 in the hospital (Bhengsri et al., 2011b). Incidence rates of meliodosis in Nakhon Panom in 2006-2008 were

estimated to be 14.9 cases per 100,000 population per year (95% confidence interval [CI], 13.3-16.6) (Bhengsri et al., 2011b). The increase in reported incidences of meliodosis in this region could also be due to increases in disease awareness, increases in diagnostic capacity, and increases in the population susceptible to *B. pseudomallei* infection due to changing demographics (more elderly people) and increasing diabetes mellitus prevalence (White, 1994). Information on the incidence of melioidosis in other provinces in Northeast Thailand is limited (Figure 1-13).

Figure 1-13 Evidence and distribution of melioidosis in Thailand from 1910 to 2015 (Hinjoy et al., 2018)

Red icons represent geolocated records of culture-confirmed human cases (1a), culture-confirmed animal cases and (1b). Green, orange, pink, rose, blue and yellow colors represent Northeast, North, East, West, South and Central Thailand, respectively. Interactive data is available at www.melioidosis.info/map.aspx



1.7.2.2 North Thailand

North Thailand covers 9 provinces (93,691 km^2), some of which share a border with Myanmar and Laos. Most meliodosis cases reported in this region are from Chiang Mai province. From 2001 to 2003, 26 culture confirmed melioidosis cases with a case fatality rate of 42% were reported from one hospital in Chang Mai (Chaiwarith, 2005). In 1997, 110 culture-confirmed melioidosis cases were observed in general and regional hospitals in the region; however, details of provinces were not specified (Vuddhakul et al., 1999). Knowledge of the epidemiology of human melioidosis in this region is limited, and it is possible that melioidosis is seldom suspected as a cause of illness in this region (Figure 1-13).

1.7.2.3 West Thailand

West Thailand is a long mountainous region, covers 5 provinces (53,679 km^2) running parallel to the border with Myanmar. Reports of human melioidosis cases from this region are rare. From 2006 to 2010, culture-confirmed meliodosis cases were reported from two provinces; Ratchaburi (n=50) and Phetchaburi (n=8) (Limmathurotsakul et al., 2012a). The estimated incidence rates were 1.20 and 0.35 per 100,000 population per year (Limmathurotsakul et al., 2012a). Animal melioidosis was also reported from Ratchaburi province (Kongkaew, 2017) (Figure 1-13), and *B. pseudomallei* has been isolated from soil in Prachuap Kiri Khan province (Finkelstein et al., 2000).

1.7.2.4 East Thailand

East Thailand consist of 7 provinces and covers an area of 34,381 km^2 (The Bureau of registration Administration, 2013). East Thailand was previously perceived to be a non-endemic area for melioidosis. In 2011, Bhengsri et al reported that *B. pseudomallei* caused

bacteraemia in Sa Kaeo province with an incidence rate of 4.9 cases per 100,000 population (95% CI =3.9-6.1) between 2006 and 2008 (Bhengsri et al., 2011b). The CFR of melioidosis in the province was 44%, which was higher than that in Nakhon Panom province (CFR of 34%) reported in the same study (Bhengsri et al., 2011b). In 2012, more human melioidosis cases were reported from another two provinces in the region; Chachoengsao and Chonburi. The study reported incidence rates of melioidosis of 24.1 and 1.2 per 100,000 population per year in Chachoengsao and Chonburi, respectively; however, the CFR was not specified (Limmathurotsakul et al., 2012a). Observed incidences of meliodosis in goats from these two provinces were 27.9 and 9.5 per 100,000 goat population per year, respectively (Limmathurotsakul et al., 2012a) (Figure 1-13).

The incidence rate of melioidosis reported from Chachoengsao province was as high as that observed in the Northeast (Limmathurotsakul et al., 2010b), suggesting that the East Thailand might be another area highly endemic for melioidosis. In addition, an environmental survey for the organism has never been conducted in the region.

1.7.2.5 South Thailand

South Thailand consists of 14 provinces, covering an area of 70,716 km^2 (The Bureau of registration Administration, 2013). The region has two long coastlines connecting to Andaman sea on the western side, and to the Gulf of Thailand on the eastern side. Sporadic culture confirmed cases have been reported from Phangnga (total of six cases) after the west coast was devastated by the 2004 tsunami (Chierakul et al., 2005b) (Figure 1-13). Suggested routes of infection were aspiration and laceration (Chierakul et al., 2005b). In 2013 one culture confirmed meliodosis case was reported from France, with a history of travel to Koh Samui (Rossi et al., 2013). In the following year, a melioidosis outbreak of 11 cases was

reported on Phangan island (Thaipadungpanit et al., 2014), which led to an environmental investigation for *B. pseudomallei* in water sources on the island. *B. pseudomallei* were isolated from multiple sources of water from the island (Thaipadungpanit et al., 2014). Both Koh Samui and Phangan island are located in Surat Thani province, where *B. pseudomallei* was isolated from soil and water in the very first environmental survey conducted in Thailand in 1964-1967 by Finkelstein et al. (Finkelstein et al., 2000). In this survey the organism was found in all 14 provinces in South Thailand (Finkelstein et al., 2000). This implies that the population of the South are at risk of melioidosis, and that incidences of melioidosis might be largely under-diagnosed and under-reported in all 14 provinces.

1.7.2.6 Central Thailand

Central Thailand consists of 21 provinces, covering an area of 93,005 km^2 (The Bureau of registration Administration, 2013). The region possess nutrient-rich soils and water sources, and agriculture such as rice farming can be performed in the region all year round. Although the first Thai culture-confirmed melioidosis case was reported from the Central region (Chittivej et al., 1995), and many cases were also reported in Bangkok (Chayasirisobhon, 1976) (Figure 1-13), most of these cases are believed to have acquired *B. pseudomallei* in the Northeast.

All soil and water samples from five provinces in Central region were negative for *B. pseudomallei* in an environmental survey in 1964-1967 (Finkelstein et al., 2000). However, Vuddhakul et al. reported a total of 127 culture-confirmed melioidosis cases in Central Thailand (Vuddhakul et al., 1999). In addition, *B. pseudomallei* has been isolated from soil in the Central region (Vuddhakul et al., 1999). Furthermore, melioidosis has been reported in goats in Bangkok, suggesting that the animals may have acquired *B. pseudomallei* from

the environment in Central Thailand (Tonpitak et al., 2014). This published evidence suggests that Central Thailand could also be endemic for melioidosis, and melioidosis might be under-diagnosed and under-reported in the region.

In summary, the current information ofnenvironmental *B. pseudomallei* does not represent the risk areas where the rice farming rural population may be exposed to the organism. A soil survey has never been conducted in East Thailand, where melioidosis is potentially also endemic. Knowledge about factors associated with the presence of environmental *B. pseudomallei* is limited to the Northeast, and cannot explain the presence or absence of *B. pseudomallei* in different regions outside Northeast Thailand. The presence of other *Burkholderia* species may be associated with the presence of *B. pseudomallei*, but this has never been evaluated systematically. Understanding factors associated with the presence of *B. pseudomallei* may also provide useful information on how to eradicate or reduce the high density of *B. pseudomallei* present in the soil in Northeast Thailand.

The true burden of melioisosis in Thailand is not known. There is a large discrepancy between numbers of cases and deaths of melioidosis reported in the national surveillance system and published data, indicating that the incidence of and mortality from melioidosis is severely under-reported in the country. This has led to my dissertation which aims to evaluate where in Thailand melioidosis is a threat to public health and an important health burden. My dissertation addresses four research questions listed in the following section.

1.8 Scope of this dissertation

My four research questions are:

(1) What is the geographical distribution of *B. pseudomallei* in Northeast, East and Central Thailand?

(2) What are the ecological factors associated with presence of environmental *B. pseudomallei*?

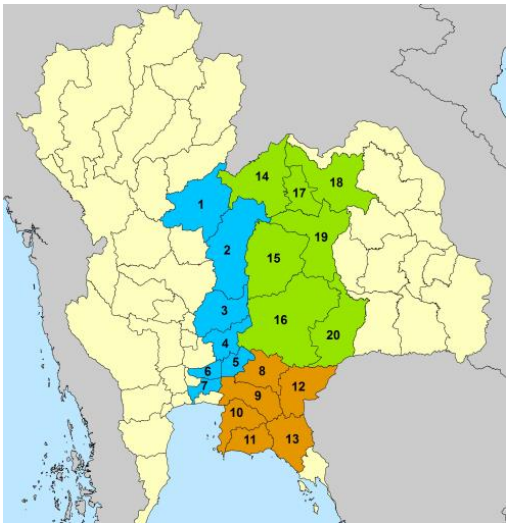
(3) What are the serological responses in people exposed to environmental *B. pseudomallei* and other neighbouring species?

(4) What is the incidence of meliodosis nationwide?.

1.8.1 What is the geographically distribution of *B. pseudomallei* in Northeast, East and Central Thailand?

With the aim of describing the geographical distribution of *B. pseudomallei* in multiple regions of Thailand and explaining the presence or absence of *B. pseudomallei* in different regions outside northeast Thailand, a large environmental survey was conducted simultaneously with an evaluation of soil physicochemical factors in the East, Central and Northeast Thailand. A total of 20 provinces were included in the study covering the East (6 provinces), Central (7 provinces) and Northeast (7 provinces) regions of Thailand (Figure 1-14). A total of 60 rice fields were evaluated (3 fields per province). Provinces in Northeast, East and Central Thailand were selected because they represent adjacent areas with different incidence rates of melioidosis. The consensus guidelines for the detection of environmental *B. pseudomallei* were used (Limmathurotsakul et al., 2013a).

Figure 1-14 Map of 20 provinces from Central, East and Northeast Thailand aimed to be sampled for presence of *B. pseudomallei*.



Central (7 provinces): (1) Phitsanulok, (2) Phetchabun, (3) Lop Buri, (4) Saraburi, (5) Nakhon Nayok, (6) Pathum Thani, (7) Bangkok.

East (6 provinces): (8) Prachin Buri, (9) Chacoengsao, (10) Chon Buri, (11) Rayong, (12) Sa Kaeo, (13) Chanthabu.

Northeast (7 provinces): (14) Loei, (15) Chaiyaphum, (16) Nakhon Ratchasima (17) Nong Bua Lamphu, (18) Udon Thani, (19) Khon Kaen, (20) Buri Ram.

1.8.2 What are the ecological factors associated with presence of environmental *B. pseudomallei*?

The soil chemical properties play important roles for survival of *B. pseudomallei*, but these are rarely evaluated in the real environmental setting (Inglis and Sagripanti, 2006). Identifying ecological factors associated with the presence of *B. pseudomallei* could have important implications for developing a public health program to control or reduce the prevalence of the organism in the environment. A study from a single province in Northeast Thailand reported that the soil physiochemical properties associated with the presence of *B. pseudomallei* were soil pH 5-8, a moisture content >10%, high chemical oxygen demand (COD) and high nitrogen concentration (Palasatien et al., 2008). A study from Laos shows that the presence of *B. pseudomallei* is associated with the elevation above sea level (Rattanavong et al., 2011). A study from Australia shows that the presence of exotics grasses, high moisture level, acidic soil, road drainage and soil colours (reddish grey and reddish yellow) are associated with the presence of *B. pseudomallei* (Kaestli et al., 2012). It

is unclear whether these factors are associated with the presence of *B. pseudomallei* in different regions in Thailand.

During the soil sampling study conducted to answer Question 1, an evaluation of soil physiochemical properties from each study field was performed to determine ecological factors associated with the presence of *B. pseudomallei* in different regions. Soil was analyzed by iLab Asia Co., Ltd. and Central Laboratory (Thailand) Co., Ltd. Soil characteristics evaluated included soil texture, pH, lime requirement, organic matter, electrical conductivity, total nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, sodium, cation exchange capacity, moisture, carbon:nitrogen ratio, cadmium and iron. The evaluation of correlation between *B. pseudomallei* positivity in rice fields and the level of each soil characteristic were performed.

1.8.3 What are the serological responses in people exposed to environmental *B. pseudomallei*?

Serological response has been used to determine exposure to *B. pseudomallei* in endemic areas. The indirect hemagglutination assay (IHA) is commonly used with titers of 1:40 or greater being considered reactive and indicating exposure to *B. pseudomallei* (Alexander et al., 1970a, Appassakij et al., 1990, Ashdown and Guard, 1984). Populations in northeast Thailand are often found to have high serological positivity by the IHA test (Kanaphun et al., 1993, Tiyawisutsri et al., 2005, Maude et al., 2012, Wuthiekanun et al., 2006a, Cheng et al., 2006, Wuthiekanun et al., 2006b). Systematic studies of the human immune response associated with presence of environmental *B. pseudomallei* and other closely related species (*B. thailandedsis*) in different regions have never been conducted. This knowledge will be

important to demonstrate the background sero-positivity to *B. pseudomallei* in different regions in Thailand.

During the soil sampling study conducted in Question 1, serological response of the rice farmers, family members and workers who are exposed to the sampled rice fields were evaluated. Five milliliters of blood was collected from the study participants after informed consent was obtained and level of antibody against *B. pseudomallei* using IHA evaluated as described previously (Chantratita et al., 2007b). Associations between presence of *B. pseudomallei* and other closely related species (*B. thailandedsis*) in rice fields and level of serological response were evaluated.

1.8.4 What is the incidence of meliodosis nationwide?

Melioidosis is one of 81 notifiable diseases in Thailand. There is a large discrepancy between the numbers of cases and deaths of melioidosis reported in the national surveillance system and in the research literature, indicating that incidence and mortality of melioidosis are severely under-reported in the country. There are many problems with the current national reporting systems; including delayed culture positivity of *B. pseudomallei*, rapid mortality of melioidosis, and the workload of the hospitals. However, identifying the true incidence of meloidosis nationwide is crucial for informing policy makers, which could lead to an improvement in diagnosis, treatment and prevention of melioidosis in the country.

Under a collaboration with BOE MoPH, Thailand, a cross-sectional retrospective, multicentre surveillance study in all 96 regional hospitals and general hospitals in Thailand was conducted. Data from the microbiology laboratory and hospital database (2012-2015) from each hospital, and from the national death registry held by the Ministry of Interior, Thailand, for the year 2012 were obtained. The study population were all patients who

admited to the study hospitals, and the hospital database was used. The incidence rate, 30-day mortality, in-hospital mortality of melioidosis in 2012-2015 was estimated in patients with culture positive for *B. pseudomallei* from any clinical specimens using the microbiology laboratory database. The mortality rate from melioidosis in 2012-2015 was evaluated using the national death registry data.

1.9 Outline of the five chapters of dissertation

The first chapter presents general background on *B. pseudomallei* and the overall epidemiology of melioidosis. The second chapter consists of background, study design and findings regarding the distribution of and factors associated with presence of *B. pseudomallei* in the soil. The third chapter consists of background, study design and findings regarding associations between the presence of *B. pseudomaleli* and another closely related species; *B. thailandensis*. This chapter also includes the association between presence of those organisms and serological responses in healthy rice farmers working in those rice fields. The fourth chapter provides the information of background, study design and findings regarding nationwide incidence and in-hospital mortality of culture-confirmed meliodosis. Finally, the fifth chapter is where implications and suggestions drawn from the findings are discussed.

Chapter 2: Geographically distribution of *B. pseudomallei* in rice fields in East, Central, and Northeast Thailand and ecological factors associated with presence of environmental *B. pseudomallei*

Published:

Hantrakun V, Rongkard P, Oyuchua M, Amornchai P, Lim C, Wuthiekanun V, Day NP, Peacock SJ, Limmathurotsakul D: Soil Nutrient Depletion Is Associated with the Presence of *Burkholderia pseudomallei*. Appl Environ Microbiol 2016, 82(24):7086-7092.

2.1 Abstract

Burkholderia pseudomallei is a soil-dwelling bacterium and the cause of melioidosis, which kills an estimated 89,000 people per year worldwide. Agricultural workers are at high risk of infection due to repeated exposure. Little is known about soil physicochemical properties associated with presence or absence of the organism. Here, we evaluated the soil physicochemical properties and presence of *B. pseudomallei* in 6,100 soil samples collected from 61 rice fields in Thailand. The presence of *B. pseudomallei* was negatively associated with the proportion of clay, proportion of moisture, level of salinity, percentage of organic matter, presence of cadmium, and nutrient levels (phosphorous, potassium, calcium, magnesium and iron). The presence of *B. pseudomallei* was not associated with the level of soil acidity (p=0.54). In a multivariable logistic regression model, presence of *B. pseudomallei* was negatively associated with the percentage of organic matter (OR=0.06; 95%CI 0.01-0.47, p=0.007), level of salinity (OR=0.06; 95%CI 0.01-0.74, p=0.03), and percentage of soil moisture (OR=0.81; 95%CI 0.66-1.00, p=0.05). Our study suggests that

in rice fields, *B. pseudomallei* thrives in those that are nutrient-depleted. Some agricultural practices result in a decline in soil nutrients, which may impact on the presence and amount of *B. pseudomallei* in affected areas.

2.2 Author summary

Burkholderia pseudomallei is an environmental Gram-negative bacillus and the cause of melioidosis. Humans acquire the disease following skin inoculation, inhalation or ingestion of the bacterium in the environment. The presence of *B. pseudomallei* in soil defines geographic regions where humans and livestock are at risk of melioidosis, yet little is known about soil properties associated with presence of the organism. We evaluated the soil properties and presence of *B. pseudomallei* in 61 rice fields in East, Central and Northeast Thailand. We demonstrated that the organism was more commonly found in soils with lower levels of organic matter and nutrients including phosphorus, potassium, calcium, magnesium and iron. We also demonstrated that crop residue burning after harvest, which can reduce soil nutrients, was not uncommon. Some agricultural practices result in a decline in soil nutrients, which may impact on the presence and amount of *B. pseudomallei* in affected areas.

2.3 Introduction

Melioidosis, an infectious disease caused by the Gram-negative bacterium *Burkholderia pseudomallei*, is an important global public health threat. An estimated 165,000 cases of human melioidosis occur each year worldwide, of which 89,000 (54%) die (Limmathurotsakul et al., 2016). The disease is highly endemic in Southeast Asia and Northern Australia (Wiersinga et al., 2012), and is predicted to be endemic but is grossly under-reported in many tropical and sub-tropical countries (Currie et al., 2008,

Limmathurotsakul et al., 2016). The crude case fatality rate for melioidosis ranges from 14% to 40% and may be as high as 70% in cases given sub-optimal antibiotic therapy (Chierakul et al., 2005a, Limmathurotsakul et al., 2010b, White et al., 1989). No licensed vaccine for melioidosis is currently available.

B. pseudomallei is a free-living organism found in soil and water (Wiersinga et al., 2012), and humans acquire the disease following skin inoculation, inhalation or ingestion of the bacterium in the environment (Limmathurotsakul et al., 2013b). In tropical developing countries, most patients are agricultural workers (typically rice farmers) with frequent contact with soil and water. Evidence-based guidelines for the prevention of melioidosis recommend that residents and visitors to melioidosis-endemic areas avoid direct contact with soil and water, and wear protective gear such as boots and gloves when in direct contact with soil and environmental water (Faa and Holt, 2002, Limmathurotsakul et al., 2013b). However, rubber boots are hot and make walking difficult in muddy rice fields, and rubber gloves are also hot and difficult to use while planting rice (Suntornsut et al., 2016). As a result, many rice farmers continue to work in rice fields without protective gear and are at high risk of melioidosis.

The presence of *B. pseudomallei* in soil defines geographic regions where humans and livestock are at risk of melioidosis, but knowledge of environmental factors associated with the presence of the organism in the natural setting is poor and conflicting. Laboratory studies using sterile soil shows that *B. pseudomallei* grows well in soil with a high percentage of moisture (Tong et al., 1996, Chen et al., 2003, Kaestli et al., 2015), high level of iron (Wang-Ngarm et al., 2014), optimal acidity (pH 4-8) (Chen et al., 2003, Wang-Ngarm et al., 2014), and high salinity (up to 4.2 dS/m) (Wang-Ngarm et al., 2014). By contrast, two cross-

sectional studies in the natural environment in Northern Australia and Northeast Thailand found that the presence of *B. pseudomallei* was negatively associated with the level of iron in soil (Thanapat et al., 2013, Baker et al., 2015), and a recent modelling study and an experimental field study suggested that the presence of *B. pseudomallei* was not associated with soil acidity (Limmathurotsakul et al., 2016, Kaestli et al., 2015). Furthermore, both negative and positive correlations between the presence of *B. pseudomallei* and soil salinity have been reported (Limmathurotsakul et al., 2016, Kaestli et al., 2015, Baker et al., 2015). Land use can affect the biodiversity of organisms in soil (Newbold et al., 2015), but there is currently no information on the association between the presence of *B. pseudomallei* and agricultural practices.

Here, we report the findings of a large cross-sectional environmental survey to determine the physicochemical characteristics of soil associated with the presence of *B. pseudomallei* in three regions in Thailand where melioidosis is considered to be highly endemic (Northeast and East) or non-endemic (Central). Our findings extend the understanding of soil properties related to environmental *B. pseudomallei*.

2.4 Materials and Methods

2.4.1 Study area

East, Central and Northeast Thailand consist of 7, 21 and 20 provinces that cover 34,381, 93,005 and 168,854 km^2, and have an estimated population in 2013 of 3.9, 18.7 and 23.3 million, respectively (The Bureau of registration Administration, 2013). Northeast Thailand is a plateau surrounded by mountain ranges, and most of the arable land consists of tropical sandy soil. East Thailand is characterized by short mountain ranges alternating with alluvial plains. Central Thailand is a large plain consisting of clay soil. Rice farming is the

predominant form of agriculture in all three regions. In Thailand, for administrative purposes each province is sub-divided into districts, sub-districts, communes and villages. The majority of the population in all three regions live in rural settings and most adults are engaged in agriculture, particularly rice farming. In 2013, land used for agriculture was 57%, 48% and 60% in East, Central and Northeast Thailand, respectively (Office of Agricultural Economics, 2013).

To evaluate environmental factors associated with the presence of *B. pseudomallei*, we selected six, seven and seven adjacent provinces in each of East, Central and Northeast Thailand, respectively (Figure 2-1). Three villages per province were randomly selected. Randomization was performed using Stata version 14.0 (StataCorp LP, College station, Texas). Soil sampling was performed in one rice field per one village. Rice fields were selected as sampling sites since rice farming is a major risk factor for melioidosis (Suntornsut et al., 2016). The sampled fields were those that had been used for rice farming for at least 12 months prior to the sampling date. Written, informed permission was obtained from land owners prior to sampling.

2.4.2 Ethical approvals

The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2013-021-01) and the Oxford Tropical Research Ethics Committee, University of Oxford (OXTREC 1013-13).

2.4.3 Soil Sampling

Soil sampling in East, Central and Northeast Thailand was performed during the dry season (from April to June) in 2013, 2014 and 2015, respectively. We used the consensus guidelines for environmental sampling described by the Detection of Environmental *B.*

pseudomallei Working Party (DEBWorP) (Limmathurotsakul et al., 2013a). In brief, each rice field was divided into a grid system, in which 100 sampling points (10 by 10) were plotted 2.5 meters apart. At each sampling point, around 30 grams of soil was removed from the base of a 30-cm hole, placed in a zip bag, and kept at ambient temperature and protected from sunlight. We recorded the location of sampled fields using the EpiCollect application (www.epicollect.net, Imperial College, London) (Aanensen et al., 2009). All soil samples were processed within 48 hours of collection for the identification of *B. pseudomallei* and for soil physicochemical properties.

2.4.4 Identification of *B. pseudomallei*

Ten grams of soil from each sampling point was mixed with 10 ml of enrichment broth consisting of threonine-basal salt solution plus colistin (TBSS-C50 broth) and incubated at 40°C in air for 48 hours. Ten microliters of surface liquid was then sub-cultured onto Ashdown agar and incubated at 40°C in air and examined every 24 hours for 4 days for bacterial colonies suggestive of *B. pseudomallei,* which were initially identified on the basis of colony morphotype. This included the characteristic colony morphology (purple, flat, dry and wrinkled) together with six additional colony morphotypes, as described previously (Chantratita et al., 2007a). Presumptive colonies were picked from each sample and tested immediately using a specific latex agglutination test for *B. pseudomallei*-specific CPS, as previously described (Anuntagool et al., 2000a, Anuntagool et al., 2000b, Wuthiekanun et al., 2002). For positive colonies, susceptibility to amoxicillin/clavulanic acid and arabinose assimilation were determined as previously described (Wuthiekanun et al., 1996). *B. pseudomallei* was defined based on the combination of colony morphology, positive latex

agglutination test, susceptibility to amoxicillin/clavulanic acid and negative arabinose assimilation (Wuthiekanun et al., 1996).

2.4.5 Soil properties

One kilogram of soil from each sampling field was made by aggregating 100 soil samples (10 g per each sampling point) and evaluated for four main properties (Table 2-1), as follows. (1) Physical properties: texture (proportion of sand, silt and clay) and moisture (% weight for weight [%w/w]). (2) Acidity and salinity: pH, lime requirement (to adjust soil acidity; kg/100sqm) and electrical conductivity (deciSiements/meter [dS/m]). (3) Chemical properties: total nitrogen (mg/kg), available phosphorous (mg/kg), exchangeable potassium (mg/kg), exchangeable calcium (mg/kg), available magnesium (mg/kg), extractable sulphur (mg/kg), total iron (g/kg), total cadmium (mg/kg), exchangeable sodium (mg/kg) and cation exchange capacity (cmol/mg). (4) Biological related factors: organic matter (%w/w) and carbon to nitrogen ratio (C:N ratio).

2.4.6 Agricultural practices

A closed-end interviewee-based questionnaire was used to collect the information about agricultural practices (Appendix 1). For illiterate participants, the questionnaire was read to the participant and completed by trained research staff in accordance with their responses. Questions included fertilizer used and rice field management (before planting and after harvest) in the 12 months before the sampling date.

2.4.7 Sample size calculation

To determine the optimal sample size, we performed a pilot study of soil sampling in four rice fields in Chachoengsao province, East Thailand. Three of four rice fields (75%) were culture positive for *B. pseudomallei*. We calculated that 60 rice fields (3 rice fields per

province) were needed to determine environmental factors associated with *B. pseudomallei* with a power of 80% at an alpha error of 5%.

2.4.8 Statistical analysis

The outcomes of interest were positivity of *B. pseudomallei* in rice fields and its association with soil properties. Binary and continuous variables were compared by using the Fisher's exact test and Mann-Whitney test, respectively. Soil properties associated with the presence of *B. pseudomallei* were evaluated using univariable and multivariable logistic regression. The final multivariable logistic regression models were developed using a purposeful selection method (Bursac et al., 2008). Sensitivity analysis was conducted using region-stratified analysis. We also used ordered logistic regression to evaluate the association between soil properties and quantity of *B. pseudomallei*. The number of positive sampling points for *B. pseudomallei* within a rice field was used to represent the quantity of *B. pseudomallei* distribution in the field. The Spearman correlation coefficient was used to evaluate the correlation between soil properties. All statistical tests were performed using Stata version 14.0 (StataCorp LP, College station, Texas). The final database with the data dictionary are publicly available online (https://figshare.com/s/b44c335a9b321ab19325).

Table 2-1 Soil physicochemical properties methods used and normal range

Soil physicochemical characteristics	Method & Definition	Normal range
Physical factors		
• Percentage of sand, silt and clay	Hydrometer method classified by particles size: sand (0.05– 2 mm), silt (0.002 – 0.05 mm) and clay (<0.002 mm)	Combination of sand, silt and clay is 100%
• Moisture (%w/w)	Gravimetric method	Not available
Acidity and salinity factors		
• pH	pH meter method: Acidity and alkalinity of the soil	Extremely acid <4.5, Very acid soil 4.5-5.5, Mild acid 5.6-6.5, Neutral 6.6–7.3, Mild alkaline 7.4-7.8, Alkaline 7.9-8.4, Very alkaline 8.5-9.0, Extremely alkaline 9.0[2]
• Electrical conductivity (dS/m)	EC meter method: soluble salt content in the soil	Very low (non-saline) 0-2, Mild salinity 2-4, saline soil 4-8, high 8-16, very high ≥16[2]
• Lime requirement (kg/100sqm)	Woodruff buffer method	Not available
Chemical factors		
• Total nitrogen (mg/kg)	Kjeldahl method	Not available
• Available phosphorous (mg/kg)	Bray II method	Very low <6, low 6-12, average 13-25, high 26-50, very high >50 mg/kg[2]
• Exchangeable potassium (mg/kg)	Automated Flame photometric method	Very low <16, low 16-30, average 31-60, high 60-120, very high >120 mg/kg[2]
• Exchangeable calcium (mg/kg)	Flame photometic method	Not available
• Available magnesium (mg/kg)	Flame photometic method	Not available
• Extractable sulphur (mg/kg)	Turbidimetric method	Not available
• Exchangeable sodium (mg/kg)	Flame photometic method 6020	Not available
• Total iron (Fe; g/kg)[3]	Based on US EPA method 6101B	Not available
• Total cadmium (mg/kg) [3]	Based on US EPA method 6020	<37 mg/kg[1]
• Cation exchange capacity (cmol/kg)	Filtration method: The number of exchangeable cations per dry weight that a soil is capable of holding, at a given pH value, and available for exchange with the soil water solution.	>15 cmol/kg[2]
Biological related factors		
• Organic matter (%w/w)	Walkley and black method.	very low <0.5%, low 0.5-1.5%, average 1.5-2.5%, somewhat high 2.5-3.5%, high 3.5-4.5%, very high >4.5%[2]
• Carbon to nitrogen ratio	Walkley and black method/Kjeldahl method: A ratio of the mass of carbon to the mass of nitrogen in a substance.	Not available

[1] Pollution Control Department (PCD), Ministry of Science Technology and Environment, Thailand
[2] Land Development Department (LDD), Ministry of Agriculture and Cooperatives, Thailand
[3] Iron and cadmium were tested by Central Laboratory (Bangkok, Thailand).

2.5 Results

2.5.1 Distribution of *B. pseudomallei* in Northeast, East and Central Thailand

Of 6,100 soil samples collected from 61 rice fields (100 soil samples per rice field), 1,046 were culture positive for *B. pseudomallei* (Figure 2-1). A total of 30 of 61 rice fields (49%) had at least one sampling point that was culture positive for the organism. Percentages of rice field culture-positive for *B. pseudomallei* were 57% (12 of 21 rice fields), 68% (13 of 19 rice fields) and 24% (5 of 21 rice fields) in Northeast, East and Central Thailand, respectively. The percentage of rice fields culture-positive for *B. pseudomallei* in the Northeast and East were higher than that in Central Thailand (57% vs. 24%, p=0.06 and 68% vs. 24%, p=0.01), while the percentage was not significantly different between the Northeast and East (57% vs. 68%, p=0.53).

For the rice fields that were culture-positive for *B. pseudomallei*, the median number of positive sampling points were 53 (range 2 to 98), 16 (range 1 to 81) and 1 (range 1 to 63) in Northeast, East and Central Thailand, respectively (Table 2-2). The median number of positive sampling points in the Northeast and East were both higher than that in Central Thailand (p=0.01 and p=0.002), while the number was not significantly different between the East and Northeast (p=0.61).

2.5.2 Characteristics of soil and agricultural practices

Overall comparison of soil properties among the three regions studied shows that soils from Central Thailand have the highest median percentage of clay (53%), followed by the Northeast (45%) and East (32%). Soil acidity (pH) varied considerably, ranging from very acid (pH=4.9) to carbonate-rich soil (pH=8.1), but was not significantly different between

the three regions (p=0.68). Soil salinity, as determined by electrical conductivity and expressed in dS/m, was very low in all fields sampled (<2.0 dS/m).

Farmers were interviewed about land management before and after rice planting (including the fertilizer used, and crop residue burning before and after harvest) in the 12 months before the sampling date. Of 61 rice fields evaluated, 54 (89%) were treated with chemical fertilizer, 17 (28%) with organic fertilizer made from plant material, 22 (36%) with organic fertilizer made from animal dung, and 39 (64%) with biological fertilizer such as "Effective Microorganisms". Owners of 24 (39%) rice fields burned their fields between rice planting seasons. The median percentage of organic matter in fields with a history of burning was not significantly lower than that of others (0.81 vs. 0.84 %w/w, p=0.82).

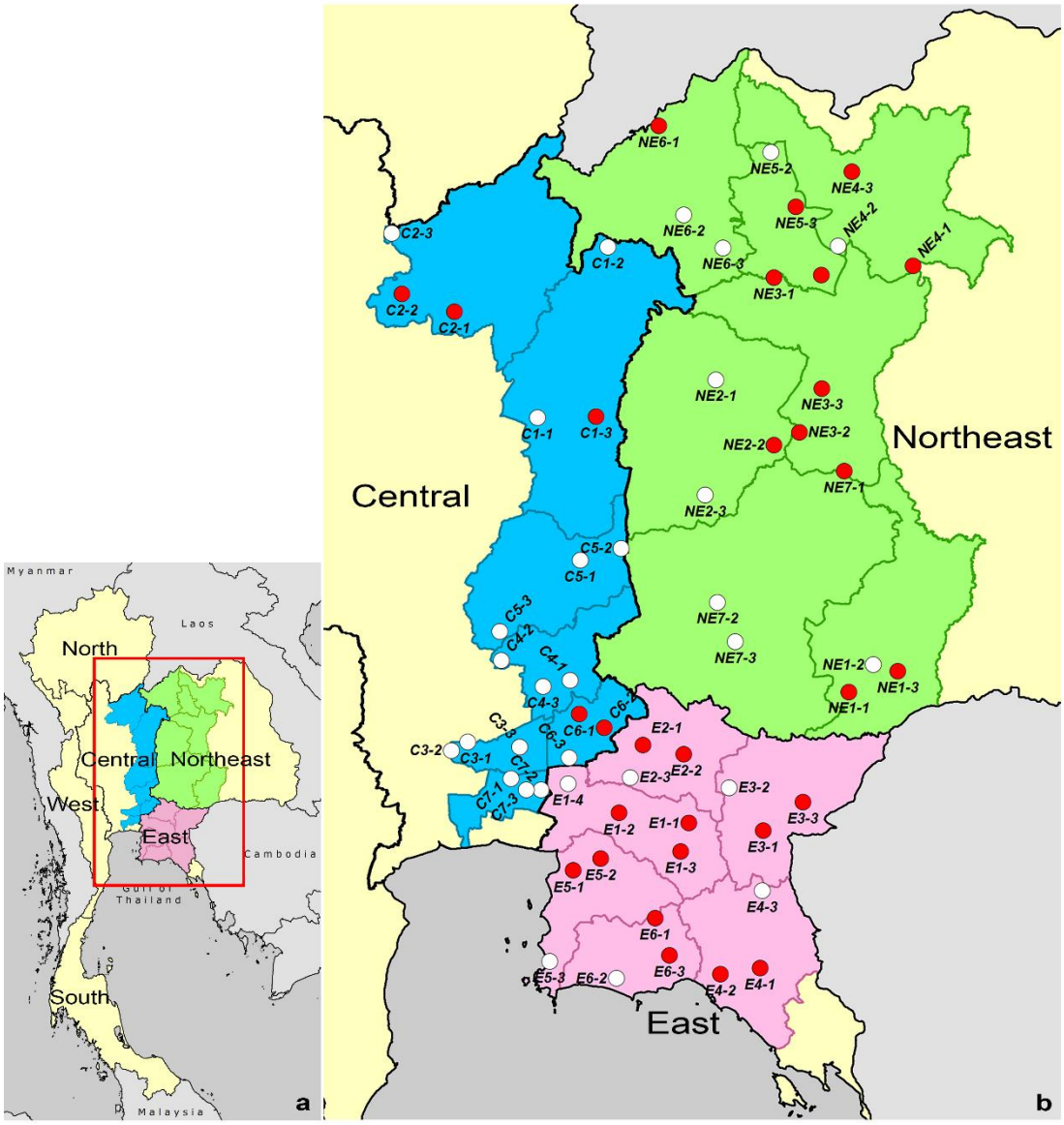
Table 2-2 Number of culture positive samples for *B. pseudomallei* in 61 rice fields

Region	Province	Number of sampling points culture positive for *B. pseudomallei* per 100 sampling points (sampled rice fields[1])			
		Rice field no. 1	Rice field no. 2	Rice field no. 3	Rice field no.4
Northeast	Burirum	87 (NE1-1)	0 (NE1-2)	97 (NE1-3)	-
	Chaiyaphum	0 (NE2-1)	98 (NE2-2)	0 (NE2-3)	-
	Khon Kaen	72 (NE3-1)	94 (NE3-2)	35 (NE3-3)	-
	Udon Thani	40 (NE4-1)	0 (NE4-2)	28 (NE4-3)	-
	Nong Bua Lam Phu	58 (NE5-1)	0 (NE5-2)	48 (NE5-3)	-
	Loei	4 (NE6-1)	0 (NE6-2)	0 (NE6-3)	-
	Nakhon Ratchasima	2 (NE7-1)	0 (NE7-2)	0 (NE7-3)	-
East	Chachoengsao[2]	39 (E1-1)	8 (E1-2)	43 (E1-3)	0 (E1-4)
	Prachinburi	10 (E2-1)	6 (E2-2)	0 (E2-3)	-
	Sa Kaeo	17 (E3-1)	0 (E3-2)	81 (E3-3)	-
	Chanthaburi	16 (E4-1)	1 (E4-2)	0 (E4-3)	-
	Chonburi	3 (E5-1)	32 (E5-2)	0 (E5-3)	-
	Rayong	1 (E6-1)	0 (E6-2)	57 (E6-3)	-
Central	Phetchabun	0 (C1-1)	0 (C1-2)	3 (C1-3)	-
	Phitsanulok	1 (C2-1)	1 (C2-2)	0 (C2-3)	-
	Pathum Thani	0 (C3-1)	0 (C3-2)	0 (C3-3)	-
	Saraburi	0 (C4-1)	0 (C4-2)	0 (C4-3)	-
	Lopburi	0 (C5-1)	0 (C5-2)	0 (C5-3)	-
	Nakhon Nayok	63 (C6-1)	1 (C6-2)	0 (C6-3)	-
	Bangkok	0 (C7-1)	0 (C7-2)	0 (C7-3)	-

1 Each rice field was divided into a grid system, in which 100 sampling points (10 by 10) were plotted 2.5 m apart. At each sampling point, 10 g of soil at 30 cm depth was collected and cultured for *B. pseudomallei*.
2A pilot study of soil sampling were performed in four rice fields in Chachoengsao province, East Thailand.

Figure 2-1 Distribution of *B. pseudomallei* in Central, East and Northeast Thailand.

(a) Map of Thailand. (b) Location of the 61 rice fields evaluated. Red and white circles, culture positive and negative for *B. pseudomallei*, respectively. Province codes represent Phetchabun (C1), Phitsanulok (C2), Pathum Thani (C3), Saraburi (C4), Lopburi (C5), Nakhon Nayok (C6) and Bangkok (C7) in Central Thailand, Chachoengsao (E1), Prachinburi (E2), Sa Kaeo (E3), Chanthaburi (E4), Chonburi (E5) and Rayong (E6) in the East, and Burirum (NE1), Chaiyaphum (NE2), Khon Kaen (NE3), Udon Thani (NE4), Nong Bua Lam Phu (NE5), Loei (NE6) and Nakhon Ratchasima (NE7) in the Northeast.



2.5.3 Association between soil physicochemical properties and *B. pseudomallei*

We found that the presence of *B. pseudomallei* was associated with nutrient-depleted soil (Table 2-3, Figure 2-2). Presence of the organism was negatively associated with the percentage of soil moisture ($p<0.001$), the level of soil salinity ($p=0.001$), presence of cadmium ($p<0.001$) and levels of multiple nutrients including available phosphorous ($p=0.03$), exchangeable potassium ($p<0.001$), exchangeable calcium ($p=0.001$), available magnesium ($p=0.002$) and total iron ($p=0.002$). Levels of overall nutrients and total nutrient fixing capacity of soil determined by organic matter and cation exchange capacity, respectively, were also negatively associated with the presence of *B. pseudomallei* (both p values<0.001). The carbon to nitrogen ratio, which is used to determine how easily bacteria can decompose organic material in soil, was also negatively associated with the presence of *B. pseudomallei* ($p=0.01$). Presence of the organism was positively associated with the proportion of sand ($p=0.02$), negatively associated with the proportion of clay ($p=0.002$), and not associated with the proportion of silt ($p=0.68$). Presence of *B. pseudomallei* was not associated with soil acidity ($p=0.54$), or agricultural practices. Many soil physicochemical properties were strongly correlated (Table 2-4).

We used multivariable logistic regression analysis and found that the presence of *B. pseudomallei* was negatively associated with the percentage of organic matter (OR=0.06; 95%CI 0.01-0.47, $p=0.007$), level of salinity (OR=0.06; 95%CI 0.01-0.74, $p=0.03$), and level of soil moisture (OR=0.81; 95%CI 0.66-1.00, $p=0.05$) (Table 2-5). A sensitivity analysis was conducted by including region as a stratification variable, which gave comparable results.

In addition, we also used ordered logistic regression to further evaluate the association between the quantity of *B. pseudomallei* distribution in rice fields and soil physicochemical factors. We observed that the number of sampling points culture positive for *B. pseudomallei* was also negatively associated with the percentage of organic matter (OR=0.06; 95%CI 0.01-0.32, p=0.001), level of soil moisture (OR=0.78; 95%CI 0.66-0.91, p=0.002) and level of salinity (OR=0.07; 95%CI 0.01-0.53, p=0.01) (Table 2-6).

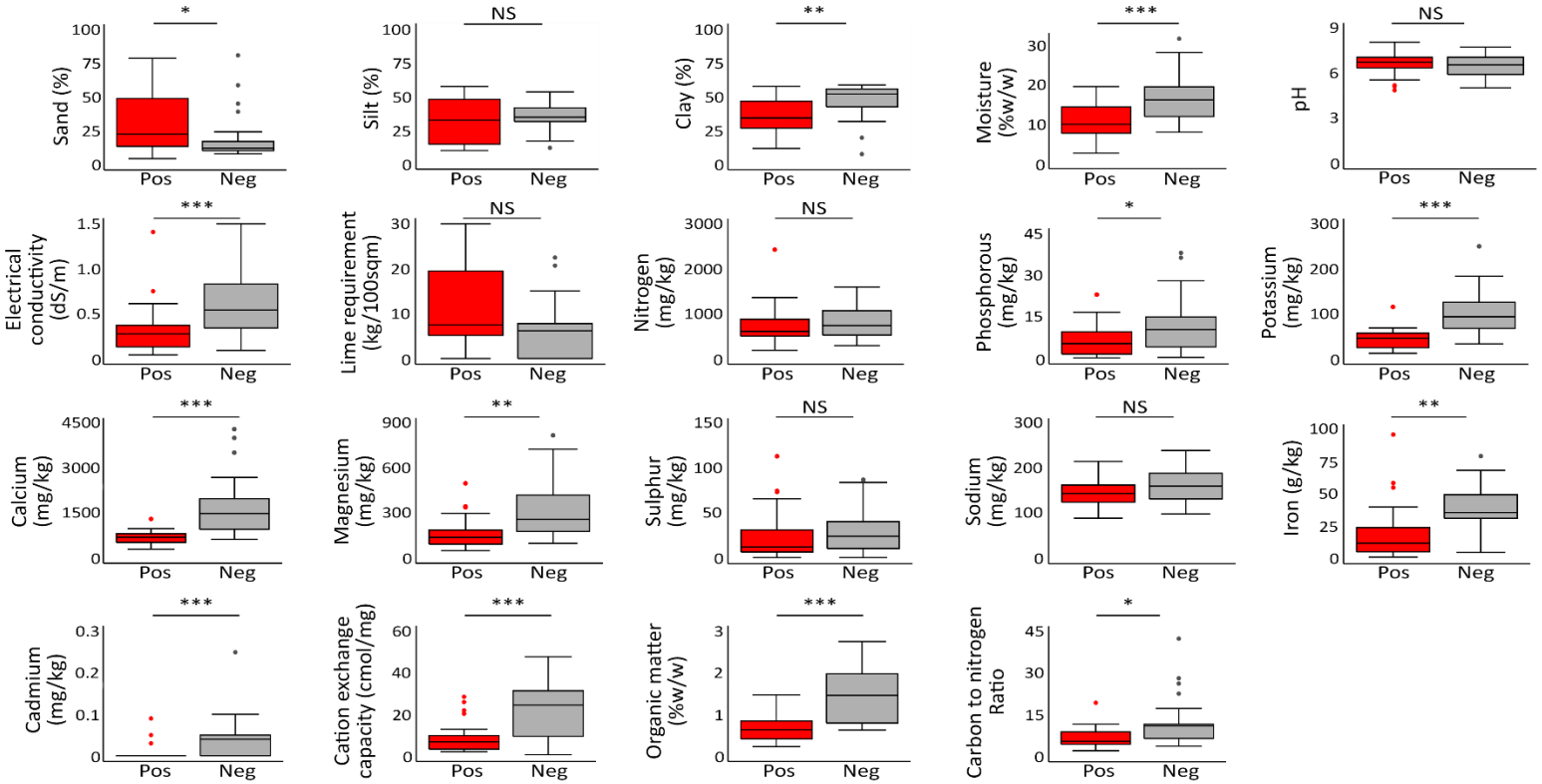
Table 2-3 Soil physicochemical properties associated with the presence of *B. pseudomallei*

Soil physicochemical characteristics	Rice fields positive for *B. pseudomallei*[1] (n=30)	Rice fields negative for *B. pseudomallei*[1] (n=31)	Crude odds ratio (95% CI)	p value
Physical factors				
• Sand (%)	22.4 (4.3-78.8)	12.0 (7.8-81.0)	1.04 (1.01-1.07)	0.02
• Silt (%)	32.2 (9.7-57.2)	34.5 (11.7-53.2)	0.99 (0.95-1.03)	0.68
• Clay (%)	34.1 (11.5-57.6)	51.9 (7.3-58.6)	0.93 (0.88-0.97)	0.002
• Moisture (%w/w)	9.9 (2.5-19.5)	16.1 (7.9-31.8)	0.78 (0.67-0.90)	<0.001
Acidity and salinity factors				
• pH	6.8 (4.9-8.1)	6.6 (5.0-7.8)	1.23 (0.63-2.40)	0.54
• Electrical conductivity (dS/m)	0.3 (0.04-1.4)	0.5 (0.1-1.5)	0.22 (0.09-0.53)	0.001
• Lime requirement (kg/100sqm)	7.5 (0-30)	6.2 (0-22.5)	1.00 (1.00-1.01)	0.10
Chemical factors				
• Total nitrogen (mg/kg)	598 (175-2,442)	731 (278-1,601)	0.99 (0.98-1.01)[2]	0.41
• Available phosphorous (mg/kg)	5 (0.2-23)	10 (0.4-38)	0.35 (0.15-0.83)[2]	0.02
• Exchangeable potassium (mg/kg)	45 (11-115)	93 (32-252)	0.52 (0.37-0.74)[2]	<0.001
• Exchangeable calcium (mg/kg)	673 (272-1,289)	1474 (602-4,326)	0.93 (0.90-0.97)[2]	0.001
• Available magnesium (mg/kg)	134 (44-498)	255 (93-823)	0.92 (0.87-0.97)[2]	0.003
• Extractable sulphur (mg/kg)	12 (0-114)	24 (0-88)	0.94 (0.77-1.15)[2]	0.55
• Exchangeable sodium (mg/kg)	144 (89-217)	161 (98-241)	0.87 (0.75-1.01)[2]	0.07
• Total iron (Fe; g/kg)	12 (0.6-96)	36 (4- 79)	0.95 (0.92-0.98)	0.002
• Total cadmium				
○ not detected	25 fields (83%)	11 fields (35%)	1.0	
○ detected	5 fields (17%)	20 fields (65%)	0.11 (0.03-0.37)[3]	<0.001
• Cation exchange capacity (cmol/mg)	6.7 (2.0-28.5)	24.4 (0.6-47.9)	0.89 (0.84-0.95)	<0.001
Biological related factors				
• Organic matter (%w/w)	0.6 (0.2-1.5)	1.5 (0.6-2.8)	0.02 (0.003-0.17)	<0.001
• Carbon to nitrogen ratio	5.4 (2.0-19.3)	11.0 (3.6-42.6)	0.81 (0.70-0.94)	0.01

[1] Median (range) unless other are specified.
[2] Odds ratio for any increase of 100 mg/kg in nutrient.

Figure 2-2 Soil physicochemical properties associated with the presence of *B. pseudomallei*

Box–whisker plots indicate median, interquartile range and distribution of the data. Dots indicate the outliers (data located outside 1.5 times of interquartile range) (Tukey, 1977).



Red and grey boxes represent rice fields culture positive (Pos) and negative (Neg) for *B. pseudomallei*, respectively. *P≤0.05, **P≤0.01, ***P≤0.001 and NS=Not Significant.

Table 2-4 Correlation coefficients among soil physicochemical properties in the East, Central and Northeast Thailand

	Sand	**Silt**	**Clay**	**pH**	**LR[1]**	**OM[1]**	**EC[1]**	**TotalN[1]**	**AvailP[1]**	**ExchK[1]**
Sand	1									
Silt	-0.74***	1								
Clay	-0.77***	0.13	1							
pH	0.12	-0.03	-0.15	1						
LR[1]	0.22	-0.29*	-0.05	-0.49***	1					
OM[1]	-0.25*	-0.01	0.38**	-0.10	-0.18	1				
EC[1]	-0.14	-0.09	0.29*	-0.10	-0.11	0.35**	1			
TotalN[1]	-0.13	-0.08	0.27*	-0.15	0.14	0.35**	0.18	1		
AvailP[1]	-0.17	0.16	0.11	-0.04	-0.01	0.37**	0.11	0.28*	1	
ExchK[1]	-0.27*	-0.11	0.51***	-0.26*	-0.16	0.63***	0.44***	0.31*	0.40**	1
ExchCa[1]	-0.19	-0.07	0.35**	-0.06	-0.36**	0.57***	0.29*	0.10	0.17	0.64***
AvailMg[1]	-0.27*	0.02	0.38**	-0.14	-0.28*	0.47***	0.19	0.16	0.003	0.47***
ExtrS[1]	-0.20	0.17	0.14	-0.20	-0.01	0.14	0.21	0.14	0.16	0.17
ExchNa[1]	-0.38**	0.26*	0.32*	-0.23	0.03	0.26*	0.12	0.16	0.24	0.29*
CEC[1]	-0.31*	-0.06	0.51***	-0.25	-0.12	0.76***	0.34**	0.27*	0.18	0.69***
Moisture	-0.46***	0.16	0.53***	-0.27*	-0.18	0.55***	0.23	0.26*	0.20	0.60***
CNRatio[1]	0.003	0.001	-0.01	0.05	-0.24	0.57***	0.04	-0.36**	0.28*	0.27*
CD[1]	-0.25*	0.12	0.26*	0.07	-0.13	0.63***	0.15	0.09	0.24	0.32*
Iron[1]	-0.38**	0.08	0.48***	-0.16	0.004	0.63***	0.11	0.21	0.27*	0.42***

Statistically significant *p≤0.05, **p≤0.01 and ***p≤0.001

Table 2-5 Soil physicochemical properties associated with the presence of *B. pseudomallei* in a multivariable logistic regression model

Soil physicochemical characteristics	Adjusted odds ratio (95% CI)	p value
Organic matter (%w/w)	0.06 (0.01-0.47)	0.007
Electrical conductivity (dS/m)	0.06 (0.01-0.74)	0.03
Moisture (%)	0.81 (0.66-1.00)	0.05

Table 2-6 Association between soil physicochemical properties and quantity of *B. pseudomallei* distribution in rice field determined by ordered logistic regression

Soil physicochemical characteristics	Adjusted odds ratio (95% CI)	p value
Organic matter (%w/w)	0.06 (0.01-0.32)	0.001
Moisture (%)	0.78 (0.66-0.91)	0.002
Electrical conductivity (dS/m)	0.07 (0.01-0.53)	0.01

2.6 Discussion

The results of our large environmental study demonstrated an association between the presence of *B. pseudomallei* and nutrient-depleted soil in rice fields in Thailand. Negative associations between the presence of *B. pseudomallei* and nutrient levels in the soil were observed for each of the nutrients evaluated (with the exception of total nitrogen, exchangeable sodium and extractable sulphur) and for organic matter and cation exchange capacity, which represent levels of overall nutrients and total nutrient fixing capacity of soil, respectively. This is also supported by the negative association between the presence of *B. pseudomallei* and the level of salinity, which could also represent the level of soil nutrients in the environment (Kaestli et al., 2015). Our findings are important because nutrients in the soil are effected by agricultural practices, and crop residue burning after harvest is not uncommon in Thailand and many other tropical countries. There is strong evidence to show that burning can reduce soil nutrients by eliminating crop residues and soil organisms present on the soil surface (FAO, 2005). Poor agricultural practices could impact on the presence and amount of *B. pseudomallei*. This suggests that changes in agricultural practice and improvement of soil nutrient content might also be essential to reduce the distribution of *B. pseudomallei* and incidence of melioidosis.

Our study also highlights the difference between findings from experimental soil inoculated with *B. pseudomallei*, environmental studies in small areas where melioidosis is endemic, and this large environmental study. For example, soil moisture was positively associated with presence of *B. pseudomallei* in experimental soil studies (Tong et al., 1996, Chen et al., 2003, Kaestli et al., 2015), and environmental studies of small areas where melioidosis is endemic (Kaestli et al., 2009, Sermswan et al., 2015). It has been postulated that *B. pseudomallei* can move from deeper soil layers to the surface during the rainy

season and rising water table where it may then multiply (Thomas et al., 1979). Our study shows that soil in Northeast Thailand (where *B. pseudomallei* is abundant in soil) is mostly sandy soil with a low level of organic matter and moisture, while soil in Central Thailand (where *B. pseudomallei* is less abundant), is mostly clay soil with high level of organic matter and moisture. This is also supported by a recent finding of the presence of *B. pseudomallei* in a desert region outside the wet tropics in Northern Australia (Yip et al., 2015).

Organic matter in soil contains vital nutrients and influences the diversity and biological activity of soil organisms (FAO, 2005). The negative association between soil organic matter and the presence of *B. pseudomallei* is consistent with two previous environmental studies in Northern Australia (Baker et al., 2015) and Northeast Thailand (Ngamsang et al., 2015), which show that the level of organic carbon is negatively associated with presence of *B. pseudomallei*. The level of organic carbon is a measure of the carbon contained within the soil organic matter. It is possible that soils with high organic matter have high biotic stress because abundant soil microorganisms are competing for substrates, water or growth factors (Ian L. Pepper, 2009), which may inhibit the survival or growth of *B. pseudomallei*. This is supported by an environmental study showing that low microbial density in soil is associated with the presence of *B. pseudomallei* (Sermswan et al., 2015, Potisap et al., 2018) and that *Bacillus amyloliquefaciens* extracted from soil samples can inhibit the growth of *B. pseudomallei* (Chotima et al., 2016). It is also possible that depletion of individual nutrients such as iron supports the growth of *B. pseudomallei,* which has a range of mechanisms to persist in low iron environments (Ribolzi et al., 2016). An additional possibility is that environmental stress selects for persister cells of *B. pseudomallei*, as has been recently shown for

Pseudomonas aeruginosa in nutrient-limited conditions and in biofilm (Nguyen et al., 2011). *B. pseudomallei* are taken up by amoebae, which in vitro are associated with survival in the presence of disinfecting agents and antimicrobial drugs (Inglis et al., 2004, Howard and Inglis, 2005), and may represent an additional survival advantage for *B. pseudomallei* in nutrient-depleted soil.

Our findings suggest that extremely low levels of salinity (such as <0.1 dS/m) may be an indirect measure of nutrient depletion in rice fields. This is because soil salinity as estimated by measuring electrical conductivity represents soluble salts of soil nutrients, including sodium, chloride, magnesium, calcium, potassium and nitrate. Our finding is consistent with an experimental study in Northern Australia (Kaestli et al., 2015), which shows that *B. pseudomallei* grows well in soil with low electrical conductivity (0.1 dS/m) but could not survive in commercial soil, which has a high level of organic based compost and high electrical conductivity (0.7 dS/m). Although a recent modelling study proposed a positive association between salinity level and presence of *B. pseudomallei*, this estimation was based on soil salinity for all land (undisturbed land, agricultural land, sports fields, etc) with an electrical conductivity ranging from 0 to >20 dS/m (Limmathurotsakul et al., 2016). It is also possible that the effect of salinity in rice fields may be different from non-rice fields; for example, garden and unused land. For example, rice fields may be intentionally flooded and drained repeatedly to reduce salinity to a very low level (<2.0 dS/m) (FAO, UN), and this could lead to the loss of water-soluble nutrients from the soil (Inthong et al., 2005, Stoate et al., 2001, Pathak et al., 2004).

B. pseudomallei can survive well in soil under laboratory condition with pH ranging from 4 to 8 (Wang-Ngarm et al., 2014), and our study supports the lack of association between presence of *B. pseudomallei* and pH.

A limitation of our study is that soil sampling was only performed during the dry season over a period of three years. We chose to sample during the dry season to control for variation in the presence of *B. pseudomallei* and soil physicochemical properties associated with seasonal changes. Recent environmental studies showed that soil properties are not different between the dry and wet season (Thanapat et al., 2013), and that changes in the presence of *B. pseudomallei* in the soil with very low salinity level (<2.0 dS/m) measured over three years were minimal (Kaestli et al., 2015). It is possible that the presence of *B. pseudomallei* in rice fields would have been generally higher if the study was conducted during the rainy season. Although the difference in percentage of organic matter between fields with and without a history of burning was not observed in our study, this could be because of the cross-sectional study design or other confounding factors. For example, some fields were burned more than 12 months before the study was conducted.

In summary, our large cross-sectional environmental survey has shown that the presence of the important human pathogen *B. pseudomallei* is associated with nutrient-depleted rice fields. Further investigations are required to evaluate whether changes in agricultural practices could effectively enhance soil nutrients, and whether these could reduce the distribution of *B. pseudomallei* in rice fields.

Chapter 3: Serological responses in people who are exposed to environmental *B. pseudomallei* and closely related species

Published:

Hantrakun V, Thaipadungpanit J, Rongkard P, Srilohasin P, Amornchai P, Langla S, Mukaka M, Chantratita N, Wuthiekanun V, Dance DAB et al: Presence of *B. thailandensis* and *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide in Thailand, and their associations with serological response to *B. pseudomallei*. PLoS Negl Trop Dis 2018, 12(1):e0006193.

3.1 Abstract

Burkholderia pseudomallei is an environmental Gram-negative bacillus and the cause of melioidosis. *B. thailandensis*, some strains of which express a *B. pseudomallei*-like capsular polysaccharide (BTCV), is also commonly found in the environment in Southeast Asia but is considered non-pathogenic. The aim of the study was to determine the distribution of *B. thailandensis* and its capsular variant in Thailand and investigate whether its presence is associated with a serological response to *B. pseudomallei*. We evaluated the presence of *B. pseudomallei* and *B. thailandensis* in 61 rice fields in Northeast (n=21), East (n=19) and Central (n=21) Thailand. We found BTCV in rice fields in East and Central but not Northeast Thailand. Fourteen fields were culture positive for *B. pseudomallei* alone, 8 for *B. thailandensis* alone, 11 for both *B. pseudomallei* and *B. thailandensis*, 6 for both *B. thailandensis* and BTCV, and 5 for *B. pseudomallei*, *B. thailandensis* and BTCV. Serological testing using the indirect hemagglutination assay (IHA) of 96 farmers who worked in the study fields demonstrated that farmers who worked in *B. pseudomallei*-positive fields had higher IHA titers than those who worked in *B.*

pseudomallei-negative fields (median 1:40 [range: <1:10-1:640] vs. <1:10 [range: <1:10-1:320], p=0.002). In a multivariable ordered logistic regression model, IHA titers were significantly associated with the presence of *B. pseudomallei* (aOR=3.7; 95% CI 1.8-7.8, p=0.001) but were not associated with presence of *B. thailandensis* (p=0.32) or BTCV (p=0.32). One sequence type (696) was identified for the 27 BTCV isolates tested. This is the first report of BTCV in Thailand. The presence of *B. pseudomallei* and *B. thailandensis* in the same field was not uncommon. Our findings suggest that IHA positivity of healthy rice farmers in Thailand is associated with the presence of *B. pseudomallei* in rice fields rather than *B. thailandensis* or BTCV.

3.2 Author summary

Burkholderia thailandensis is a non-pathogenic soil-dwelling bacterium and is genetically closely related to *Burkholderia pseudomallei*, the cause of melioidosis. In mouse models, inoculation of a variant of *B. thailandensis* which express a *B. pseudomallei*-like capsular polysaccharide (BTCV) induces antibodies and partial protection against melioidosis. Here, we evaluated the presence of *B. pseudomallei*, *B. thailandensis*, and BTCV in 61 rice fields in Northeast, East and Central Thailand, determined whether they co-existed, and if their presence was associated with a serological response in farmers. We report the presence of BTCV in Thailand for the first time and describe the distribution of the three organisms. Co-localization between these organisms in the same rice fields was not uncommon. Our findings suggested that serological positivity based on the indirect hemagglutination assay (a test commonly used to detect antibodies to *B. pseudomallei*) in healthy rice farmers in Thailand was associated with exposure to *B. pseudomallei*, but not exposure to *B. thailandensis* or BTCV.

3.3 Introduction

Burkholderia pseudomallei is a soil-dwelling Gram-negative bacterium and the cause of melioidosis, a frequently fatal infectious disease of humans and animals. Humans acquire the disease following skin inoculation, inhalation or ingestion of the bacterium from the environment. The disease is highly endemic in Southeast Asia and Northern Australia (Wiersinga et al., 2012), and is increasingly being reported in South Asia, Africa and Central and South America (Redondo et al., 2011, Currie et al., 2008). A recent modeling study estimated that there are about 165,000 human melioidosis cases per year worldwide, of whom 89,000 (54%) die (Limmathurotsakul et al., 2016). The current diagnostic standard for melioidosis is microbiological culture (Hoffmaster et al., 2015). However, melioidosis is difficult to diagnose due to its diverse clinical manifestations, the inadequacy of conventional bacterial identification methods, and a lack of microbiology laboratories in tropical developing countries (Hoffmaster et al., 2015). An indirect haemagglutination assay (IHA) is the most frequently used serological test for melioidosis, but may be misleading when used for the diagnosis of melioidosis in disease-endemic regions (Hoffmaster et al., 2015). This is because the background seropositivity (IHA titers ≥1:160) ranges from 4% to 32% in healthy individuals living in areas where melioidosis is endemic (Maude et al., 2012, Hii et al., 2016, Suttisunhakul et al., 2016). Therefore, IHA is recommended as a serological standard to assess exposure to *B. pseudomallei* (Hoffmaster et al., 2015).

Burkholderia thailandensis was first recognized by Wuthiekanun et al. in 1996 (Wuthiekanun et al., 1996). The organism is genetically closely related to *B. pseudomallei*, can be isolated from environmental soil and water, and is non-pathogenic (Wuthiekanun et al., 1996, Trakulsomboon et al., 1997, Sermswan et al., 2015). The colony morphology

of *B. thailandensis* and *B. pseudomallei* are very similar, but *B. thailandensis* can assimilate L-arabinose (Smith et al., 1997, Brett et al., 1998). In addition, *B. thailandensis* has polysaccharide-related genes that are distinct from *B. pseudomallei* (74.8% and 72.8% nucleotide and protein similarity, respectively) and usually lacks the virulence-associated capsular polysaccharide (also referred to as CPS or CPS-I) of *B. pseudomallei* (Reckseidler et al., 2001, Smith et al., 1993, Wuthiekanun et al., 2002, Sim et al., 2010). The geographical distribution of *B. thailandensis* is uncertain but the organism has rarely been isolated from fields that are culture positive for *B. pseudomallei* (Vuddhakul et al., 1999, Trakulsomboon et al., 1999). It was recently shown that *B. pseudomallei* can inhibit the growth and motility of *B. thailandensis* in the laboratory (Ngamdee et al., 2015). However, previous environmental studies did not systematically evaluate the presence of both organisms, so the presence of *B. thailandensis* and co-localization of both organisms may have been underestimated (Vuddhakul et al., 1999, Trakulsomboon et al., 1999). In an experimental mouse model, lipopolysaccharide extracted from *B. thailandensis* induced measurable IgG and IgM, and provided partial protection against melioidosis (Ngugi et al., 2010). The association between exposure to environmental *B. thailandensis* and IHA seropositivity in humans is still largely unknown.

A variant of *B. thailandensis* originally isolated from soil in Cambodia (E555) that contained genes encoding a *B. pseudomallei*-like capsular polysaccharide cluster (BTCV) was described in 2010 (Sim et al., 2010). This organism exhibited several *B. pseudomallei*-like phenotypes including colony wrinkling, resistance to human complement binding, and intracellular macrophage survival. However, in mice E555 was avirulent (Sim et al., 2010), induced higher levels of IgG and gave better protection against melioidosis than non-capsulated *B. thailandensis* (Scott et al., 2013). The capsular polysaccharide (CPS)

biosynthesis gene cluster of E555 and that of *B. pseudomallei* are highly similar (94.4% and 96% nucleotide and protein similarity, respectively) (Sim et al., 2010), and nuclear magnetic resonance spectroscopy has shown that the structures of CPS produced by E555 and that of *B. pseudomallei* are identical (Bayliss et al., 2017). Previously, BTCV has been isolated from human blood in the USA in 2003 (strain CDC3015869; ST101, USA (Glass et al., 2006)) and from environmental samples in Cambodia in 2010 (strain E555; ST696), Gabon in 2013 (strain D50; ST1126 (Wiersinga et al., 2015)) and Laos in 2015 (strain ST_10; ST696 (Knappik et al., 2015)). BTCV has not been reported in Thailand and its distribution is unknown.

We recently reported the presence of *B. pseudomallei* in 61 rice fields in the Northeast, East and Central Thailand, and its association with soil physicochemical properties (Hantrakun et al., 2016). Here, we report the presence of *B. thailandensis* and co-localization between *B. pseudomallei* and *B. thailandensis* in the same rice fields, and provide the first report of the BTCV in Thailand. In addition, we explored whether exposure to *B. thailandensis* and BTCV is associated with background seropositivity to *B. pseudomallei* by evaluating IHA levels in healthy adults who worked in the sampled rice fields.

3.4 Materials and Methods

3.4.1 Study area

East, Central and Northeast Thailand consist of 7, 21 and 20 provinces, cover 34,381, 93,005 and 168,854 km2, and had estimated populations in 2013 of 3.9, 18.7 and 23.3 million, respectively (The Bureau of Registration Administration, 2013). Northeast Thailand is a plateau surrounded by mountain ranges, and most of the arable land consists of tropical sandy soil. East Thailand is characterized by short mountain ranges alternating

with alluvial plains. Central Thailand is a large plain consisting of clay soil. Rice farming is the predominant form of agriculture in all three regions. In Thailand, for administrative purposes each province is sub-divided into districts, sub-districts, communes and villages. The majority of the population in all three regions live in rural settings and most adults are engaged in agriculture, particularly rice farming. In 2013, areas used for agriculture were 57%, 48% and 60% in East, Central and Northeast Thailand, respectively (Office of Agricultural Economics, 2013).

3.4.2 Study design

We conducted a cross-sectional environmental survey as described previously (Hantrakun et al., 2016). All *B. pseudomallei*, *B. thailandensis* and BTCV reported in this work were from the same environmental survey (Hantrakun et al., 2016). In brief, we collected soil from randomly-selected rice fields in the East, Central and Northeast regions during the dry season (from April – June) in 2013, 2014 and 2015, respectively. We sampled rice fields that had been used for rice farming in the 12 months prior to the sampling date. We collected the blood from farmers who were exposed to the sampled rice fields in the 12 months prior to the blood collection date.

3.4.3 Ethics statement

Written, informed consent was obtained from land owners and farmers prior to soil sampling and blood collection, respectively. The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2013-021-01) and the Oxford Tropical Research Ethics Committee, University of Oxford (OXTREC 1013-13).

3.4.4 Soil sampling and soil properties

The method used for soil sampling was described previously (Limmathurotsakul et al., 2013a, Hantrakun et al., 2016). In brief, each rice field was divided into a 10x10 grid system to generate 100 sampling points per field. At each sampling point, around 30 grams of soil was removed from the base of a 30-cm hole. All soil samples were processed within 48 hours of collection for the identification of *B. pseudomallei* and *B. thailandensis*, and for soil physicochemical properties. One kilogram of soil from each sampling field was made by aggregating 100 soil samples (10 g per each sampling point) and evaluated for soil physicochemical properties as described previously (Hantrakun et al., 2016).

3.4.5 Identification of *B. pseudomallei* and *B. thailandensis*

Ten grams of soil from each sampling point was mixed with 10 ml of enrichment broth consisting of threonine-basal salt solution plus colistin 50mg/L (TBSS-C50 broth) and incubated at 40°C in air for 48 hours. Ten microliters of surface liquid was then streaked onto Ashdown agar containing gentamicin 8mg/L and crystal violet 5mg/L using a calibrated loop, incubated at 40°C in air, and examined every day for 4 days for bacterial colonies suggestive of *B. pseudomallei* or *B. thailandensis* (Limmathurotsakul et al., 2012b, Chantratita et al., 2007a). *B. pseudomallei* can have seven colony morphotypes on Ashdown agar (Chantratita et al., 2007a), and cannot readily be distinguished from the colonies of *B. thailandensis* (Wuthiekanun et al., 1996, Brett et al., 1998, Smith et al., 1997). For each soil specimen, a total of up to five presumptive colonies of *B. pseudomallei* or *B. thailandensis* were picked and evaluated by a latex agglutination test that is highly specific for *B. pseudomallei* CPS (Wuthiekanun et al., 2002, Anuntagool et al., 2000b, Anuntagool et al., 2000a), and the L-arabinose assimilation test. *B. pseudomallei* was defined based on a positive latex agglutination test and negative L-

arabinose assimilation. *B. thailandensis* was defined based on a negative latex agglutination test and positive L-arabinose assimilation (Wuthiekanun et al., 2002, Wuthiekanun et al., 1996, Sim et al., 2010). Colonies which had positive results for both the latex agglutination and L-arabinose assimilation tests were defined as BTCV, as previously described (Sim et al., 2010). The monoclonal antibody used in the latex agglutination test has been shown to be positive for *B. pseudomallei* and BTCV (strains E555 and CDC3015869), and negative for *B. thailandensis* (Duval et al., 2014, Nualnoi et al., 2017).

3.4.6 Genotyping of BTCV

Because BTCV was found in rice fields in East and Central Thailand, we randomly selected three isolates of BTCV from each culture positive field and genotyped these using multilocus sequence typing (MLST) (Godoy et al., 2003). The alleles at each of the seven loci were assigned by comparing the sequences to those on the *B. pseudomallei* MLST website (https://pubmlst.org/bpseudomallei/).

Information and the sequence type of BTCV reported in this work has been deposited in the global MLST database (https://pubmlst.org/bpseudomallei/).

3.4.7 Serological response

Blood samples were collected from rice farmers who had worked in the sampled rice fields in the 12 months prior to the sampling date. The detection of antibodies against *B. pseudomallei* was performed using the IHA, as described previously (Ileri, 1965, Alexander et al., 1970b). The antigen used in the IHA was derived from a pool of two clinical *B. pseudomallei* isolates, 199a and 207a, obtained from melioidosis patients in Ubon Ratchathani, Thailand. The negative control was pooled sera from three patients with no detectable IHA titers. The positive control was pooled sera from three patients

with known positive IHA. In this study, for binary comparisons, an IHA titer of <1:80 was defined as negative based on the previous report that healthy US donor could have IHA titers up to 1:40 (Suttisunhakul et al., 2015).

3.4.8 Sample size calculation

To determine the optimal sample size, we performed a pilot study of soil sampling in four rice fields in Chachoengsao province, East Thailand. Three and four rice fields were culture positive for *B. pseudomallei* (75%; 3 of 4) and *B. thailandensis* (100%; 4 of 4), respectively. We calculated that 60 rice fields (3 rice fields per province) and 60 rice farmers were needed to determine environmental factors associated with presence of *B. pseudomallei* and *B. thailandensis,* and evaluate the association between IHA titers and presence of both organisms, respectively, with a power of 80% at an alpha error of 5%.

3.4.9 Statistical analysis

We evaluated (1) positivity of *B. thailandensis* and BTCV in the rice fields, (2) co-localization and correlation between *B. pseudomallei, B. thailandensis* and BTCV in the rice fields, (3) soil properties associated with presence of *B. thailandensis* and BTCV, and (4) association between IHA levels and presence of those organisms in the rice fields. Fisher's exact test and Mann-Whitney test were performed to compare binary and ordinal variables, respectively. McNemar's test was used to compare the presence of two organisms. We assessed co-localization between organisms in the rice fields by using Kappa value. The Kappa value was used to describe the agreement of presence and absence of the organism in rice fields, beyond that caused by chance, as follows: 0.00–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; 0.81–1.00, high (Viera and Garrett, 2005). A Spearman rank correlation coefficient (Spearman's rho) was used to assess correlations among the total number of sampling points culture-positive for *B.*

pseudomallei, *B. thailandensis* and BTCV in the rice fields. Spearman's rho close to 0 indicates no correlation, while Spearman's rho close to 1 (or -1) indicates a strong positive (or negative) correlation between the organisms (Mukaka, 2012). We used ordered logistic regression to determine the associations between IHA level and presence of the three organisms. As we sampled more than one rice farmer per field, the analysis was stratified by rice field. Multivariable ordered logistic regression models were developed using a purposeful selection method (Bursac et al., 2008). In brief, a univariable ordered logistic regression model was used to preliminarily evaluate the crude association between presence of *B. pseudomallei*, *B. thailandensis* and BTCV and IHA titers. We decided *a priori* to evaluate their independent associations in a multivariable ordered logistic regression model, and presence of *B. pseudomallei*, *B. thailandensis* and BTCV were all included in the final multivariable model. Sensitivity analysis was conducted by grouping *B. thailandensis* and BTCV as *B. thailandensis*. All statistical tests were performed using STATA version 14.0 (StataCorp LP, College Station, Texas). The final database with the data dictionary are publicly available online (https://doi.org/10.6084/m9.figshare.4928993).

3.5 Results

3.5.1 Distribution of *B. thailandensis* and BTCV

Of 6,100 soil samples collected from 61 rice fields, 826 (14%) were culture positive for *B. thailandensis*. The percentages of rice fields that were culture-positive for *B. thailandensis* were 29% (6 of 21 rice fields), 63% (12 of 19 rice fields) and 57% (12 of 21 rice fields) in Northeast, East and Central Thailand, respectively (Figure 3-1). There was borderline evidence that culture-positivity for *B. thailandensis* was higher in the East than the Northeast (63% vs. 29%, p=0.055), while there was no significant difference between

East and Central (63% vs. 57%, p=0.76) or Northeast and Central regions (29% vs. 57%, p=0.12).

For the rice fields that were culture-positive for *B. thailandensis*, the median numbers of positive sampling points were 6.5 (range 1 to 27), 26.5 (range 1 to 100) and 10.5 (range 1 to 85) in Northeast, East and Central Thailand, respectively (Table 3-1). There was a trend towards the median number of positive sampling points for *B. thailandensis* being lower in the Northeast than the East (p=0.08), while there was no significant difference between Central versus Northeast or East Thailand (p=0.40 and 0.52, respectively).

BTCV was isolated from 11 of 61 (18%) rice fields in the East (7 fields) and Central (4 fields) regions but it was not isolated from rice fields in Northeast Thailand (Figure 3-1). Overall, the proportion of fields positive for the BTCV was lower than that for *B. thailandensis* (18% vs. 49%, p<0.001). The percentage of rice fields that were culture positive for BTCV in the East and Central regions was not significantly different (37% [7/19] vs. 19% [4/21], p=0.29). The median numbers of positive sampling points in the East and Central regions were also not significantly different (3 [range 1 to 24] vs. 4 [range 1 to 8], p= 0.45).

3.5.2 Co-localization and correlation between presence of *B. pseudomallei*, *B. thailandensis* and BTCV in the same rice fields

We previously reported the isolation of *B. pseudomallei* from 30 of 61 rice fields included in this study (Hantrakun et al., 2016). Figure 3-2 shows the number of rice fields from which *B. pseudomallei*, *B. thailandensis* and BTCV were isolated. Of 61 rice fields, 14 (23%) were positive for *B. pseudomallei* alone, 8 (13%) positive for *B. thailandensis* alone, 11 (18%) positive for both *B. pseudomallei* and *B. thailandensis*, 6 (10%) positive for *B. thailandensis* and BTCV, and 5 (8%) positive for *B. pseudomallei*, *B. thailandensis*

and BTCV. Co-localization of *B. pseudomallei* and *B. thailandensis* in the same rice field was not more frequent than expected by chance (Kappa value 0.08, p=0.26). The numbers of sampling points per rice field that were culture positive for *B. pseudomallei* and for *B. thailandensis* were not correlated (Spearman's rho -0.02, 95%CI -0.27 to 0.23, p=0.89). A sensitivity analysis was conducted by considering BTCV as *B. thailandensis*, which gave a comparable result (Spearman's rho -0.02, 95%CI -0.27 to 0.23, p=0.87). All eleven fields culture positive for BTCV were also culture positive for *B. thailandensis* (Figure 3-2). There was a fair agreement between presence of *B. thailandensis* and BTCV (Kappa value 0.37, p<0.001), and a strong correlation between the total number of sampling points culture positive for the two organisms (Spearman's rho 0.68, 95% CI 0.51 to 0.79, p<0.001).

3.5.3 Co-localization and correlation between presence of *B. pseudomallei*, *B. thailandensis* and BTCV in the same sampling points

Of 6,100 soil samples collected, 975 (16%) were positive for *B. pseudomallei* alone, 706 (12%) positive for *B. thailandensis* alone, 24 (0.4%) positive for BTCV alone, 69 (1%) positive for both *B. pseudomallei* and *B. thailandensis*, 1 (0.02%) positive for *B. pseudomallei* and BTCV, 50 (0.8%) positive for *B. thailandensis* and BTCV, and 1 (0.02%) positive for *B. pseudomallei*, *B. thailandensis* and BTCV. There was a slight agreement between presence of *B. thailandensis* and BTCV in the same soil sample (Kappa value 0.09, p<0.001). Co-localization of *B. pseudomallei* and *B. thailandensis* in the same soil sample was also not greater than that expected by chance (p>0.99).

3.5.4 Soil physicochemical properties associated with presence of *B. thailandensis* and BTCV

Associations between soil physicochemical properties and the presence of *B. thailandensis* were not observed (Table 3-2). Presence of BTCV was negatively associated with cation exchange capacity, which represents the total nutrient fixing capacity of soil (p=0.05), and associated with the level of total nitrogen (p=0.04; Table 3-3). The associations were also observed in the multivariable model (Table 3-4).

3.5.5 Genetic diversity of BTCV

A total of 27 isolates of BTCV from 76 culture positive sampling points for BTCV in 11 rice fields in East and Central Thailand were randomly selected for MLST (Table 3-5). All 27 isolates belonged to sequence type (ST) 696, which was identical to the ST of BTCV strain E555 reported from soil in Cambodia (Sim et al., 2010). We had previously reported a single *B. pseudomallei* isolate (strain A-330-05-1-04) from drinking water in Ubon Ratchathani, northeast Thailand, as ST696 (Limmathurotsakul et al., 2014). The isolate was re-evaluated. The isolate was found to be positive for both latex agglutination and L-arabinose assimilation and was thus re-classified as BTCV.

Figure 3-1 Map of the presence of *B. pseudomallei, B. thailandensis* and *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide (BTCV) in 61 rice fields in Northeast (n=21), East (n=19) and Central (n=21) Thailand

(a) Map of Thailand. (b) Location of the 61 rice fields evaluated. Red, green and yellow pies represent rice fields that were culture positive and negative for *B. pseudomallei*, *B. thailandensis*, and BTCV, respectively. Province codes represent Burirum (NE1), Chaiyaphum (NE2), Khon Kaen (NE3), Udon Thani (NE4), Nong Bua Lam Phu (NE5), Loei (NE6) and Nakhon Ratchasima (NE7) in the Northeast, Chachoengsao (E1), Prachinburi (E2), Sa Kaeo (E3), Chanthaburi (E4), Chonburi (E5) and Rayong (E6) in the East, Phetchabun (C1), Phitsanulok (C2), Pathum Thani (C3), Saraburi (C4), Lopburi (C5), Nakhon Nayok (C6) and Bangkok (C7) in Central Thailand. ArcGis Version 10.2 (ESRI, Redlands, CA, USA) was used to map the sampled rice fields. The location of sampled rice fields was recorded by using the EpiCollect application (www.epicollect.net, Imperial College, London).

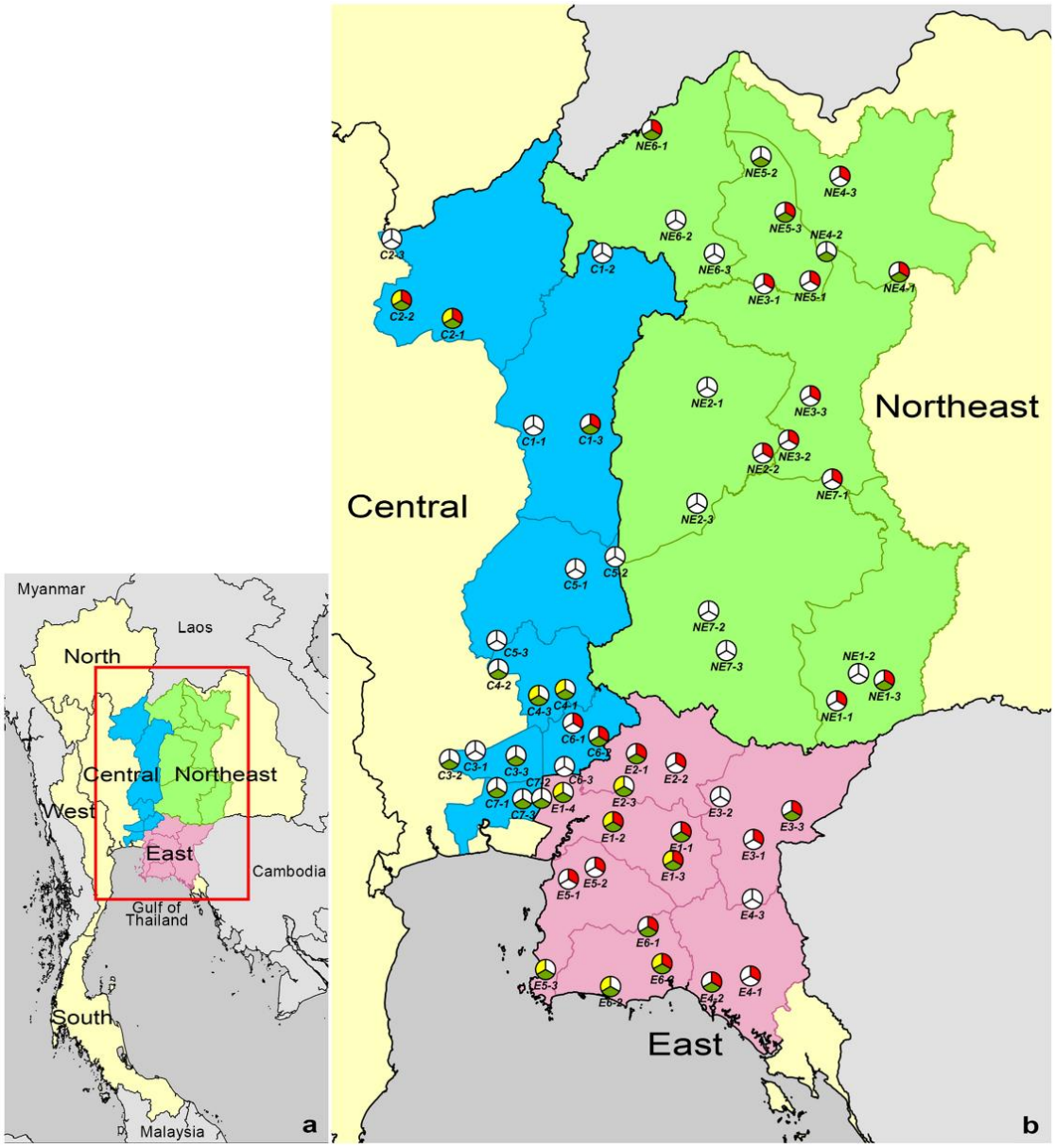


Table 3-1 Number of culture-positive sampling points for *B. pseudomallei* (*B. ps*), *B. thailandensis* (*B. th*) and *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide variant (BTCV) in 61 rice fields in the Northeast (n=21), East (n=19) and Central (n=21) Thailand

Regions	Provinces	Field[1]	Number of sampling points culture positive for		
			B. ps	*B. th*	BTCV
Northeast	Buri Rum	NE1-1	87	0	0
		NE1-2	0	0	0
		NE1-3	97	1	0
	Chaiyaphum	NE2-1	0	0	0
		NE2-2	98	0	0
		NE2-3	0	0	0
	Khonkaen	NE3-1	72	0	0
		NE3-2	94	0	0
		NE3-3	35	0	0
	Udonthani	NE4-1	40	20	0
		NE4-2	0	27	0
		NE4-3	28	0	0
	Nong Bua Lamphu	NE5-1	58	0	0
		NE5-2	0	6	0
		NE5-3	48	7	0
	Loei	NE6-1	4	6	0
		NE6-2	0	0	0
		NE6-3	0	0	0
	Nakhon Ratchasima	NE7-1	2	0	0
		NE7-2	0	0	0
		NE7-3	0	0	0
East	Chachoengsao[2]	E1-1	39	29	0
		E1-2	8	70	1
		E1-3	43	33	16
		E1-4	0	24	3
	Prachin Buri	E2-1	10	2	0
		E2-2	6	0	0
		E2-3	0	16	10
	Sa Kaeo	E3-1	17	0	0
		E3-2	0	0	0
		E3-3	81	1	0
	Chanthaburi	E4-1	16	0	0
		E4-2	1	16	0
		E4-3	0	0	0
	Chon Buri	E5-1	3	0	0
		E5-2	32	0	0
		E5-3	0	44	3

Regions	Provinces	Field[1]	Number of sampling points culture positive for		
			B. ps	***B. th***	**BTCV**
	Rayong	E6-1	1	11	0
		E6-2	0	100	24
		E6-3	57	54	2
Central	Phetchabun	C1-1	0	0	0
		C1-2	0	0	0
		C1-3	3	7	0
	Phitsanulok	C2-1	1	74	2
		C2-2	1	70	8
		C2-3	0	0	0
	Pathum Thani	C3-1	0	0	0
		C3-2	0	2	0
		C3-3	0	2	0
	Saraburi	C4-1	0	85	6
		C4-2	0	1	0
		C4-3	0	26	1
	Lop Buri	C5-1	0	0	0
		C5-2	0	0	0
		C5-3	0	0	0
	Nakhon Nayok	C6-1	63	0	0
		C6-2	1	65	0
		C6-3	0	0	0
	Bangkok	C7-1	0	6	0
		C7-2	0	13	0
		C7-3	0	8	0

[1]Each rice field was divided into a grid system, in which 100 sampling points (10 by 10) were plotted 2.5 m apart. At each sampling point, 10 g of soil at 30 cm depth was collected and cultured for *B. pseudomallei*, *B. thailandensis* and BTCV. [2]A pilot study of soil sampling were performed in four rice fields in Chachoengsao province, East Thailand.

Figure 3-2 Overlap between presence of *B. pseudomallei* (*B. ps*; red), *B. thailandensis* (*B. th*; green) and *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide (BTCV; yellow) in 61 sampled rice fields

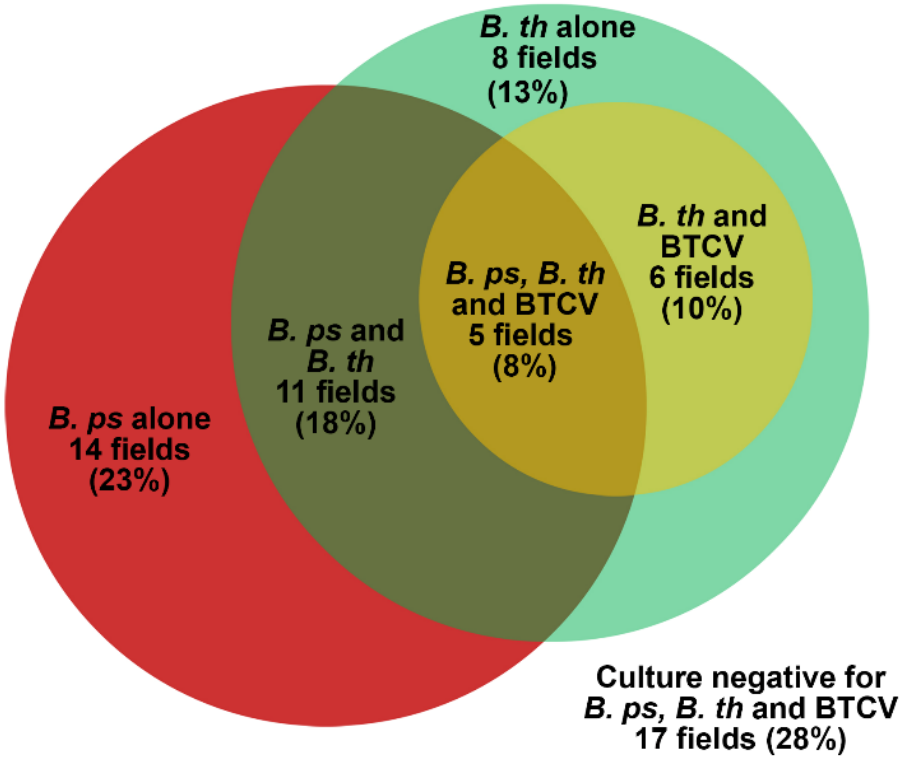


Table 3-2 Soil physicochemical properties associated with the presence of *B. thailandensis* in univariable logistic regression models

Soil physicochemical characteristics	Rice fields positive for *B. thailandensis*[1] (n=30)	Rice fields negative for *B. thailandensis*[1] (n=31)	Crude odds ratio (95% confidence interval)	p value
Physical factors				
• Sand (%)	13.2 (4.3-78.8)	18.2 (7.8-81.0)	0.98 (0.96-1.01)	0.19
• Silt (%)	34.8 (9.7-56.3)	33.1 (11.1-57.2)	1.03 (0.99-1.07)	0.16
• Clay (%)	46.7 (11.5-58.6)	45.2 (7.3-57.6)	1.01 (0.97-1.05)	0.54
• Moisture (%w/w)	14.6 (2.5-28.3)	12.8 (4.0-0.0)	1.05 (0.96-1.15)	0.32
Acidity and salinity factors				
• pH	6.6 (4.9-7.8)	6.8 (5.0-8.1)	0.54 (0.27-1.09)	0.09
• Electrical conductivity (dS/m)	0.3 (0.0-1.5)	0.4 (0.0-1.2)	1.14 (0.28-4.59)	0.85
• Lime requirement (kg/100sqm)	7.5 (0.0-30.0)	5.4 (0.0-23.0)	1.01 (0.95-1.08)	0.73
Chemical factors				
• Total nitrogen (mg/kg)	682 (330-2442)	643 (175-1,380)	1.01 (0.99-1.03)[2]	0.10
• Available phosphorous (mg/kg)	8.1 (0.6-28)	5.6 (0.2-38)	1.11 (0.60-2.04)[2]	0.75
• Exchangeable potassium (mg/kg)	57.5 (13.5-184)	58.2 (10.5-252)	0.98 (0.87-1.09)[2]	0.65
• Exchangeable calcium (mg/kg)	808 (494-2,182)	945 (272-4,326)	0.99 (0.99-1.00)[2]	0.11
• Available magnesium (mg/kg)	212 (44-616)	169 (47-823)	1.00 (0.97-1.03)[2]	0.85
• Extractable sulphur (mg/kg)	19 (2.2-114)	12 (0-74)	1.19 (0.96-1.48)[2]	0.12
• Exchangeable sodium (mg/kg)	144 (94-241)	161 (88.5-241)	0.91 (0.79-1.05)[2]	0.21
• Total iron (Fe; g/kg)	24 (1.9-96)	33 (0.6-79.6)	0.98 (0.77-1.24)[2]	0.87
• Total cadmium				
o not detected	19 fields (63%)	17 fields (55%)	1.0	
o detected	11 fields (37%)	14 fields (45%)	0.70 (0.27-1.84)	0.50
• Cation exchange capacity (cmol/mg)	7.6 (0.6-44)	12 (2-48)	0.99 (0.95-1.04)	0.75
Biological related factors				
• Organic matter (%w/w)	0.8 (0.2-2.4)	0.9 (0.2-2.8)	0.78 (0.35-1.72)	0.53
• Carbon to nitrogen ratio	6.3 (2.0-22.6)	9.5 (2.3-42.6)	0.92 (0.83-1.02)	0.10

[1] Median (range) unless otherwise specified.
[2] Odds ratio for an increase of 100 mg/kg in nutrient.

Table 3-3 Soil physicochemical properties associated with the presence of *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide (BTCV) in univariable logistic regression models

Soil physicochemical characteristics	Rice fields positive for BTCV[1] (n=11)	Rice fields negative for BTCV[1] (n=50)	Crude odds ratio (95% confidence interval)	p value
Physical factors				
• Sand (%)	14.7 (9.8-42.1)	15.1 (4.3-81.0)	0.97 (0.93-1.02)	0.22
• Silt (%)	32.3 (29.3-56.3)	34.6 (9.7-57.2)	1.03 (0.98-1.09)	0.27
• Clay (%)	45.5 (27.3-58.6)	45.6 (7.3-57.6)	1.02 (0.97-1.08)	0.41
• Moisture (%w/w)	15.4 (10.4-19.5)	12.9 (2.5-0.0)	1.05 (0.94-1.18)	0.35
Acidity and salinity factors				
• pH	6.6 (5.1-7.8)	6.7 (4.9-8.1)	0.67 (0.29-1.57)	0.35
• Electrical conductivity (dS/m)	0.2 (0.1-1.5)	0.4 (0.0-1.4)	0.71 (0.11-4.76)	0.72
• Lime requirement (kg/100sqm)	7.6 (5.8-22.5)	6.0 (0.0-30.0)	1.05 (0.97-1.14)	0.22
Chemical factors				
• Total nitrogen (mg/kg)	792.0 (330-2,442)	599.9 (175-1,601)	1.02 (1.00-1.03)[2]	0.04
• Available phosphorous (mg/kg)	12.2 (4-28)	6.1 (0.2-38)	1.95 (0.95-4.00)[2]	0.07
• Exchangeable potassium (mg/kg)	57 (31.7-114.8)	58 (10.5-252)	0.93 (0.79-1.10)[2]	0.41
• Exchangeable calcium (mg/kg)	841 (696-1,680)	822 (272-4,326)	0.99 (0.98-1.00)[2]	0.28
• Available magnesium (mg/kg)	193 (123-292)	174 (44-823)	0.98 (0.94-1.03)[2]	0.42
• Extractable sulphur (mg/kg)	18 (4.3-75)	18 (0-114)	1.01 (0.78-1.31)[2]	0.95
• Exchangeable sodium (mg/kg)	144 (108-241)	151 (88.5-241)	1.02 (0.85-1.22)[2]	0.83
• Total iron (Fe; g/kg)	3 (0.4-5.9)	2.8 (0.1-9.6)	0.97 (0.71-1.33)	0.85
• Total cadmium				
o not detected	6 fields (55%)	30 fields (60%)	1.0	
o detected	5 fields (45%)	20 fields (40%)	1.25 (0.34-4.65)	0.74
• Cation exchange capacity (cmol/mg)	4.7 (0.6-21.9)	13 (2-48)	0.92 (0.85-1.00)	0.05
Biological related factors				
• Organic matter (%w/w)	0.8 (0.3-1.5)	0.9 (0.2-2.8)	0.47 (0.13-1.61)	0.23
• Carbon to nitrogen ratio	5.6 (2.0-11.7)	9.1 (2.3-42.6)	0.81 (0.65-1.00)	0.05

[1] Median (range) unless otherwise specified.
[2] Odds ratio for an increase of 100 mg/kg in nutrient.

Table 3-4 Soil physicochemical properties associated with the presence of *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide in a multivariable logistic regression model

Soil physicochemical characteristics	Adjusted odds ratio (95% confidence interval)	p value
Total nitrogen (mg/kg)	1.04 (1.01 - 1.07) [1]	0.01
Cation exchange capacity (cmol/mg)	0.86 (0.76 - 0.97)	0.01

[1] Odds ratio for an increase of 100 mg/kg in nutrient.

Table 3-5 Reports of *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide worldwide from 1921 to 2016.

Year	Strains	Sequence type (allele profile)[1]	Sources	References
2003	CDC3015869	101 (6-5-9-11-14-20-14)	Blood of a 2-year-old male presenting with drowning, post-cardiac arrest, pneumonia and septicemia in Texas, USA	(Glass et al., 2006)
2008	E555	696 (6-5-9-11-7-20-14)	Soil in Cambodia	(Sim et al., 2010)
2012	A-330-05-1-04	696	Water (tap water) in Ubon Ratchathani, northeast Thailand [2]	(Limmathurotsakul et al., 2014)
2012-2013	D50	1126 (6-5-9-5-7-7-5)	Soil in Gabon	(Wiersinga et al., 2015)
2013	ST_10	696	Water in Laos	(Knappik et al., 2015) and personal communication: DABD and Dr Sabine Dittrich
2013-2014	SBXCB001a, SBXCB002a, SBXCB003a, SBXCC001a, SBXCC002a, SBXCC005a, SBXCC008a, SBXCC014b, SBXCC019a, SBXCC020a, SBXPL001a, SBXPL002a, SBXPL005a, SBXPL007a, SBXPL010a, SBXPR001a, SBXPR002a, SBXPR005a, SBXRY001a, SBXRY017a, SBXRY019a, SBXRY030a, SBXRY031a, SBXSR003a, SBXSR004a, SBXSR005a, SBXSR007a	696	Soil in East and Central Thailand	This study

[1] The *B. pseudomallei* MLST allele profile corresponds to the gene order *ace-gltB-gmhD-lepA-lipA-narK-ndh* . [2] Previously reported as *B. pseudomallei*.

3.5.6 IHA titers and their association with presence of *B. pseudomallei*, *B. thailandensis* and BTCV

Of 96 rice farmers included in the analysis, 29, 35 and 32 were from Northeast, East and Central Thailand, respectively. The median number of farmers per rice field was 1 (range 1 to 5). Sixty-two farmers (65%) were male and median age was 51 years (range 23-75 years). Six farmers (6%) had a known diagnosis of diabetes. Overall, 27 (28%) farmers had a positive IHA (IHA titers ≥1:80). Forty eight farmers who worked in rice fields culture-positive for *B. pseudomallei* had higher IHA titers than the 48 farmers who worked in rice fields culture-negative for the organism (median 1:40 [range: <1:10-1:640] vs. <1:10 [range: <1:10-1:320], p=0.002) (Figure 3-3). Proportion of farmers who had positive IHA (IHA ≥1:80) was also significantly higher in rice field culture-positive for *B. pseudomallei* than the farmers who work in rice field culture-negative for *B. pseudomallei* (44% vs. 13%, p=0.001).

In the univariable ordered logistic regression model, IHA titers were associated with the presence of *B. pseudomallei* (OR=3.39; 95% CI 1.66-6.90, p=0.001) but not with presence of *B. thailandensis* (OR=0.92; 95% CI 0.44-1.90, p=0.82) or BTCV (OR=1.04; 95% CI 0.60-1.80, p=0.89). A multivariable ordered logistic regression model was used to evaluate independent association between IHA titers of rice farmers and presence of each organism. IHA titers were independently associated with the presence of *B. pseudomallei* (aOR=3.72; 95% CI 1.76-7.84, p=0.001) but not associated with the presence of *B. thailandensis* (p=0.32) or BTCV (p=0.32, Table 3-6).

Figure 3-3 IHA titers associated with the presence of *B. pseudomallei*, *B. thailandensis* and *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide (BTCV) in the rice fields, respective

Box-and-whisker plots indicate median, interquartile range and distribution of IHA titers. Dots indicate outliers (data located outside 1.5 times of interquartile range). (a) IHA titers of farmers whose rice fields were culture positive for *B. pseudomallei* alone (22 farmers). (b) IHA titers of farmers whose rice fields were culture positive for *B. pseudomallei* and either *B. thailandensis* or BTCV (26 farmers), (c) IHA titers of farmers whose rice fields culture positive for either *B. thailandensis* or BTCV (22 farmers), and (d) IHA titers of farmers whose rice fields culture negative for *B. pseudomallei*, *B. thailandensis* and BTCV (26 farmers).

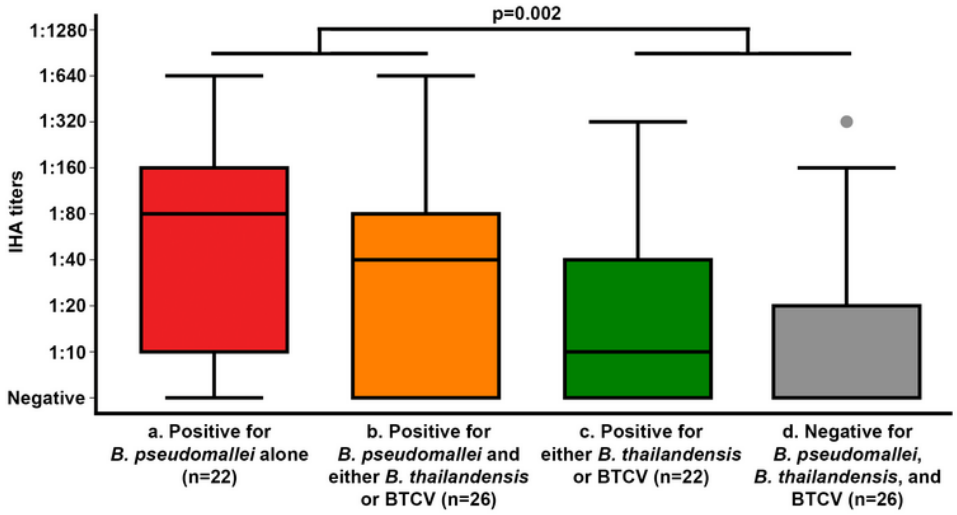


Table 3-6 Factors associated with indirect hemagglutination assay (IHA) results in 96 healthy rice farmers

Organisms cultured from rice fields	IHA results		Odds Ratios (95% confidence interval)[1]	
	IHA positive[2] (n=27 famers)	IHA negative[2] (n=69 farmers)	Univariable analysis	Multivariable analysis
B. pseudomallei	21/27 (78%)	27/69 (39%)	3.3 (1.66-6.90), p=0.001	3.72 (1.76-7.84), p=0.001
B. thailandensis	12/27 (44%)	36/69 (52%)	0.92 (0.44-1.91), p=0.82	0.63 (0.25-1.57), p=0.32
BTCV	4/27 (15%)	17/69 (25%)	1.04 (0.60-1.80), p=0.89	1.62 (0.63-4.17), p=0.32

[1] Estimated by ordered logistic regression models stratified by sampled rice field.
[2] IHA titers ≥ 1:80 is defined as positive; IHA titers <1:80 is defined as negative.

3.6 Discussion

Here, we present data on the spatial distribution of *B. pseudomallei*, *B. thailandensis* and BTCV in Northeast, East and Central Thailand. This is the first study to report the isolation of BTCV from soil in Thailand, although we did find that a Thai water isolate previously identified as *B. pseudomallei* was actually an example of BTCV (Limmathurotsakul et al., 2014). *B. thailandensis* was commonly isolated in all three regions, while BTCV was less common but associated with *B. thailandensis*. Co-localization of *B. thailandensis* and *B. pseudomallei* was not uncommon. Our findings also suggest that IHA positivity of healthy rice farmers was associated with exposure to *B. pseudomallei* rather than to *B. thailandensis* or BTCV. This supports the recommendation that IHA could be used to measure exposure to environmental *B. pseudomallei* (Hoffmaster et al., 2015), even in areas containing other closely related *Burkholderia* species.

Our finding of co-localization of *B. pseudomallei* and *B. thailandensis* is consistent with a previous environmental study in Khon Kaen, northeast Thailand (Sermswan et al., 2015). *B. thailandensis* has been reported from many melioidosis-endemic countries; including Thailand, Laos, Vietnam (Godoy et al., 2003), Cambodia (https://pubmlst.org/bpseudomallei/), Australia (Levy et al., 2008), Papua New Guinea (Warner et al., 2008), Kenya (Godoy et al., 2003) and Gabon (Wiersinga et al., 2015). This is probably highly influenced by the locations of melioidosis research groups. *B. thailandensis* has also been reported in two non melioidosis-endemic countries; France and the United States (Godoy et al., 2003, Glass et al., 2006). *B. thailandensis* strain 82172 (ST73) was isolated from the intestine of a foal in France in 1982, while *B. thailandensis* strain CDC2721121 (ST73) was isolated from the pleural wound of a 76 year old male from Louisiana, USA, in 1997 (Godoy et al., 2003, Glass et al., 2006). As strain CDC2721121 was isolated from a

wound sample its pathogenicity in humans cannot be assumed and this strain was avirulent in a mouse model (Scott et al., 2013).

Although BTCV was common in both Central and East Thailand, its prevalence is lower than that of wild-type *B. thailandensis*. Our analysis suggested that BTCV was associated with soil with low cation exchange capacity and high levels of total nitrogen. Although Sim *et al*. raised the possibility that acquisition of the *B. pseudomallei*-like CPS in E555 might improve its environmental fitness (Sim et al., 2010), this is not supported by our findings. It is also possible that the environment we studied is not representative of the environmental niche which induced *B. thailandensis* to acquire the *B. pseudomallei*-like gene cluster.

The finding that all BTCV isolates obtained from different geographical areas in Thailand, Laos and Cambodia were ST696 suggests that these may have arisen from a single ancestor. BTCV isolates in USA (ST101) (Glass et al., 2006) and Gabon (ST1126) (Wiersinga et al., 2015) are single- and triple-locus variants of ST696, respectively (TableS5). Previous phylogenetic analysis suggested that ST101 and ST696 are closely related and possibly share the same ancestor (Sim et al., 2010). Studies using whole genome sequencing of BTCV and *B. thailandensis* from different regions are required to further understand the genetic diversity and evolution of this organism.

Our results suggest that exposure to environmental *B. thailandensis* and BTCV makes a limited contribution to IHA seropositivity in farmers. In animal models, antibodies can be detected after intraperitoneal inoculation of *B. thailandensis* and BTCV (Scott et al., 2013). Intraperitoneal inoculation can lead to rapid dissemination of *B. thailandensis* or BTCV by bypassing natural host defenses (Scott et al., 2013), and induces a serological response. Nonetheless, human exposure to *B. thailandensis* and BTCV in the natural environment rarely if ever leads to infection, unlike exposure to *B. pseudomallei.* This is also supported by the finding that intra-nasal inoculation of

high dose *B. thailandensis* or BTCV (10^6 colony forming unit [CFU]) did not cause death in BALB/c mice with rapid bacterial clearance and no visible abscess formation in sacrificed mice, whilst the LD50 of *B. pseudomallei* was less than 300 CFU in the same experiment (Sim et al., 2010).

Our study has several limitations. Soil sampling was performed during the dry season over a period of three years. We chose to sample during the dry season to control the variation in presence of the three organisms, human exposure and soil physicochemical properties associated with seasonal changes. It is possible that the presence of *B. thailandensis* and BTCV could vary according to the season. Farmers may work in multiple rice fields, and be exposed to *B. pseudomallei* in untested fields. Our study may have also detected more positive samples for *B. thailandensis* and BTCV if more than five colonies had been tested from each sampling point or using other identification methods such as PCR.

In summary, our large cross-sectional environmental survey has defined the distribution of *B. thailandensis* and BTCV in Thailand. This is the first report of BTCV in Thailand, which appears to be less common than wild-type *B. thailandensis*. Our findings also suggest that exposure to *B. thailandensis* or BTCV in the environment makes a limited contribution to IHA positivity amongst healthy farmers.

Chapter 4: Burden of melioidosis nationwide, limitation and rectification of national notifiable disease-surveillance data

In preparation of manuscript:

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4.1 Abstract

Melioidosis, an infectious disease caused by Gram-negative bacillus *Burkholderia pseudomallei*, is an important cause of death in Thailand. However, only about 10 melioidosis deaths are formally reported to the National Notifiable Disease Surveillance System (Report 506) of Ministry of Public Health (MoPH), Thailand, yearly. Therefore, priority setting for melioidosis is limited.

We conducted a large retrospective study to examine incidence and mortality of melioidosis cases already diagnosed by clinical microbiology laboratories in Thailand. Information was obtained from the microbiology and hospital database of large public hospitals in Thailand, and the Report 506 from 2012 to 2015. The national death registry in 2012 was obtained from the Ministry of Interior, Thailand to evaluate 30-day mortality.

Of 96 regional or general hospitals in Thailand, 71 (74%) participated in the study. A total of 1735, 1757, 1932 and 1702 culture-confirmed melioidosis cases were diagnosed in 2012, 2013, 2014 and 2015, respectively. The incidence rate was highest in Northeast, followed by East, North, Central, South and West Thailand. In 2012, 30-day mortality of melioidosis was 40% (696/1,735), while only 4 fatal cases of melioidosis were reported from the study hospitals through the Report 506 reporting system of MoPH. Age distribution, proportions of patients presenting with bacteraemia and pneumonia, and proportion of patients with diabetes were different by region (all p values<0.001).

Melioidosis is an important cause of death in Thailand, but not officially reported to MoPH. Clinical presentations of melioidosis may be different by region. Data from the national notifiable disease-surveillance system in resource-limited settings needs to be verified and supplemented by integrating information from readily available databases.

4.2 Introduction

Melioidosis is an infectious disease caused by Gram-negative environmental bacterium *Burkholderia pseudomalei* (Wiersinga et al., 2018). The disease is considered highly endemic in northeast Thailand and northern Australia, where *B. pseudomallei* is commonly found in soil and surface water. Skin inoculation, ingestion and inhalation are important routes of *B. pseudomallei* infection. Diabetes is the most common risk factor. The majority of patients present with sepsis

with or without pneumonia or localized abscesses. The case fatality rate (CFR) for melioidosis ranges from 14% to 40% and may be as high as 70% in cases given sub-optimal antibiotic therapy (Chierakul et al., 2005a, Limmathurotsakul et al., 2010b, White et al., 1989). A modelling study estimated that there are about 165,000 melioidosis cases per year worldwide, of which 89,000 (54%) die (Limmathurotsakul et al., 2016).

Melioidosis is difficult to diagnose due to a lack of specific clinical manifestations and of diagnostic microbiological laboratories in tropical developing countries, where the disease is endemic (Wiersinga et al., 2018). The gold standard for the diagnosis of melioidosis is culture. *B. pseudomallei* is not part of the normal human flora, and its isolation from any clinical sample is regarded as diagnostic of melioidosis. However, even with good microbiological laboratories, *B. pseudomallei* could initially be discarded as a contaminant or misidentified as other organisms such as *Pseudomonas* spp. The most widely used serological test for melioidosis is an indirect haemagglutination assay (IHA), which detects crude antibodies raised against *B. pseudomallei*. Nonetheless, the international concensus guideline recommends that the IHA should not be used to diagnose melioidosis as the test is neither sensitive nor specific in the disease-endemic regions (Hoffmaster et al., 2015). A positive result of IHA can be used to imply an exposure to environmental *B. pseudomallei* (Hoffmaster et al., 2015).

Although capacity and utilization of microbiological laboratories in Thailand is high (Teerawattanasook et al., 2017), burden and epidemiology of melioidosis in the country remain poorly understood. The National Communicable Disease Surveillance system (Report 506) was established in Thailand in 1968, and melioidosis has been one of the diseases for notification since 2002. However, only about 10 melioidosis deaths were formally reported to the Report 506 each year, thus limiting priority setting by the Ministry of Public Health (MoPH), Thailand (Hinjoy et

al., 2018). Publications on melioidosis in Thailand are mostly from few research centers in northeast Thailand (Limmathurotsakul et al., 2016). Recent environmental studies show that *B. pseudomallei* is present in the environment in other regions in Thailand; including Central, East and South Thailand, (Finkelstein et al., 2000, Vuddhakul et al., 1999, Limmathurotsakul et al., 2014, Thaipadungpanit et al., 2014, Sermswan et al., 2015, Thanapat et al., 2013, Hantrakun et al., 2018), suggesting that melioidosis might be endemic in those regions but under-reported. In this study, our objectives were to determine incidence and mortality of melioidosis cases already diagnosed by clinical microbiological laboratories in Thailand using multiple sources of data including routine microbiology and hospital admission databases from all regional and provincial hospitals, and to compare these with the national death registry from the Ministry of Interior and the Report 506 of MoPH, Thailand.

4.3 Materials and methods

4.3.1 Study area

In 2012, Thailand had a population of 64.4 million, consisted of 77 provinces and covered 513,120 km^2. The country can be divided into six regions: Northeast (21.6 million population; 20 provinces); North (6.1 million population; 9 provinces); East (4.4 million population; 7 provinces); West (3.2 million population; 5 provinces), South (9.0 million population; 14 provinces); and Central (19.8 million population; 21 provinces), based on geographical and scientific purposes (Kashino, 2014). The MoPH is responsible for health care service delivery and finance, and disease prevention and control. In March 2001, the Thai health care system was majorly reformed by introducing a universal coverage (UC), to improve equity of healthcare accessibility to all Thai citizens (NHSO, 2010). There are multiple levels of health care facilities in Thailand. In each province, there are primary care units (PCU) located in sub-districts, district hospitals, and at least

one general or regional hospital. These health care units deliver health care services to people living within their catchment areas. In 2012, there were a total of 28 regional hospitals and 68 general hospitals in Thailand (MoPH, 2012). These general and regional hospitals act as referral hospitals for smaller PCUs and district hospitals for severely ill patients. These hospitals, unlike PCUs and district hospitals, are equipped with a microbiology laboratory capable of performing bacterial culture using standard methodologies for bacterial identification and susceptibility testing provided by the Bureau of Laboratory Quality and Standards, MoPH, Thailand (Opartkiattikul and Bejrachandra, 2002).

4.3.2 Study design

We conducted a retrospective, multicentre surveillance study in all general and regional hospitals in Thailand. From the hospitals that agreed to participate, data were collected from microbiology and hospital databases between January 2012 and December 2015. Hospital number (HN) and admission number (AN) were used as a record linkage between the two databases and to identify individuals who had repeat admissions. The death registry of Thailand in 2012 was obtained from the Ministry of Interior (MoI), Thailand, and used to identify patients who were discharged from hospital but died at home shortly after, which is a common practice in Thailand (Kanoksil et al., 2013, Hongsuwan et al., 2014).

4.3.3 Ethical approvals

Ethical permission for this study was obtained from Institute for the Development of Human Research Protection, Ministry of Public Health (IHRP 2334/2556), the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2014-017-01) and the Oxford Tropical Research Ethics Committee, University of Oxford (OXTREC 521-13). Written consent was given by the directors of the hospitals to use their routine hospital database for research. Consent was

not sought from the patients as this was a retrospective study, and the Ethical and Scientific Review Committees approved the process.

4.3.4 Data collection

The microbiology laboratory data collected included hospital number (HN), admission number (AN), specimen type, specimen date and culture result. Hospital admission data were collected from the routine in-patient discharge report, which is regularly completed by attending physicians and reported to the MoPH, Thailand, as part of the national morbidity and mortality reporting system. The data collected included HN, AN, national identification 13-digit number, admission date, discharge date and diagnosis. Diagnosis in the hospital admission data were recorded using the 10th revision Thai Modification (Edition 2012) of the International Classification of Disease (ICD-10-TM) codes (Thai Health Coding Center, MoPH., 2012). Date of death was also extracted from this data. We consulted with study hospitals when the data were unclear. Data collected from the national death registry obtained from the MoI included the national identification 13-digit number and the date of death. The Report 506 data was obtained from the BOE, MoPH. The data variables include total number of cases, total number of deaths, type of healthcare facilities and provinces.

4.3.5 Definitions

Melioidosis was defined as culture positive for *B. pseudomallei* from any type of clinical specimens. Comorbidities, including diabetes mellitus, hypertension, chronic renal failure, chronic obstructive pulmonary disease (COPD), chronic liver disease, human immunodeficiency virus (HIV), tuberculosis, thalassemia, and malignancy were determined using ICD-10 codes Thailand modified version (Table 4-1). Bacteraemia was defined as blood culture positive for *B. pseudomallei*. Pneumonia was determined using ICD-10 codes or sputum culture positive for the

organism. Urinary tract infection was defined as urine culture positive for *B. pseudomallei*. Hepatosplenic abscess, septic arthritis and osteomyelitis were determined using ICD-10 codes.

30-day mortality was determined on the basis of a record of death within 30 days of the admission as recorded in the hospital admission data or by a record of death in the national death registry. In-hospital mortality was determined by using discharge status recorded in the hospital admission data. In the event that a patient had more than one episode of admission due to culture-confirmed melioidosis, only the first episode was included in the study.

4.3.6 Statistical analysis

The outcomes of interest were incidence and 30-day mortality, and their associations with regions, comorbidities and clinical manifestations. Binary and continuous variables were compared by using the Chi-square test and Kruskal-Wallis test, respectively. Risk factors associated with mortality were evaluated using univariable and multivariable logistic regression. The final multivariable logistic regression models were developed using a purposeful selection method (Bursac et al., 2008). Poisson regression models were used to assess change of incidence rates over time and to compare mortality rate among six regions. The models were stratified by hospitals. A sensitivity analysis was done by evaluating factors associated with in-hospital mortality. All statistical analyses were performed using Stata version 15.0 (StataCorp LP, College station, Texas).

Table 4-1 List of ICD-10 codes used to determine co-morbidities and clinical manifestations

Factors	ICD10 codes
Comorbidities:	
• Diabetes mellitus	E10-14
• Hypertension	I10, I15.8-I15.9
• Chronic kidney disease	N18.1-N18.5, 189
• COPD	J42, J43, J44.0-J44.1, J44.8-J44.9, J47
• Liver disease	K70, K72-K74, K75.1-K76, K77, B18.0, B18.10, B18.19, B18.2, B18.8, B18.9
• HIV	B20-B23, B24
• Pulmonary tuberculosis	A15.0-A15.9, A16.0-A16.9, O98.0, P37.0, B90.9
• Thalassemia	D56.0-D56.4, D56.8-D56.9
• Malignancy	C00-C75, C81-C96, C76-C80, C97
Clinical manifestations:	
• Pneumonia	B01.2, B05.2, B20.6, J10.0, J11.0, J12.0-J12.2, J12.8-J12.9, J13-J14, J15.0-J15.7, J15.81, J15.88, J16.8, J17.0-J17.3, J17.8, J18.0-J18.2, J18.8-J18.9, J22, J44.0, J85.1-J85.2, P23.0-P23.6, and P23.8-P23.9
• Hepatosplenic abscesses	A06.4, D73.3 and K75.0
• Septic arthritis	M00.0-M00.2 and M00.8-M01.0
• Ostomyelitis	M46.2, M86.0, M86.1-M86.6 and M86.8-M86.9

4.4 Results

Of 96 regional or general hospitals in Thailand, 95 hospitals (99%) agreed to participate in the study (Figure 4-1). Data of 24 hospitals (24%) were excluded from analysis because either microbiological laboratory data or hospital admission data obtained was incomplete. The 71 hospitals included in the analysis were located in 62 provinces (Figure 4-2). Of 71 hospitals, 50 (70%) had data for 4 years, 2 (3%) had data for 3 years, 9 (13%) had data for 2 years, and 10 (14%) had data for 1 year (Table 4-2).

A total of 8,113,051 admission records from 6,240,514 patients were evaluated. Total of 7,626 admission records had at least one clinical sample culture positive for *B. pseudomallei.* Multiple admissions with culture positive for *B. pseudomallei* were noted in 421 patients. Only the first episode of culture-confirmed melioidosis in 7,126 patients were included in further analysis.

4.4.1 Incidence of melioidosis

The average incidence rate of melioidosis during the 4-year study period was 4.06 per 100,000 population per year (Figure 4-3, Table 4-3). The total number of cases diagnosed in Northeast, Central, South, East, North, and West Thailand were 5,475, 536, 374, 364, 358 and 19 cases, respectively. The incidence rate was significantly different by region ($p<0.001$). The incidence rate was highest in Northeast Thailand (8.73 per 100,000 population per year) and lowest in West Thailand (0.18 per 100,000 population per year) (Table 4-3). The number of cases diagnosed in 2012, 2013, 2014 and 2015 were 1,735, 1,757, 1,932, and 1,702 cases, respctively (Table 4-4), and a temporal trend in incidence rates was not observed ($p=0.58$).

Figure 4-1 Flow chart of study

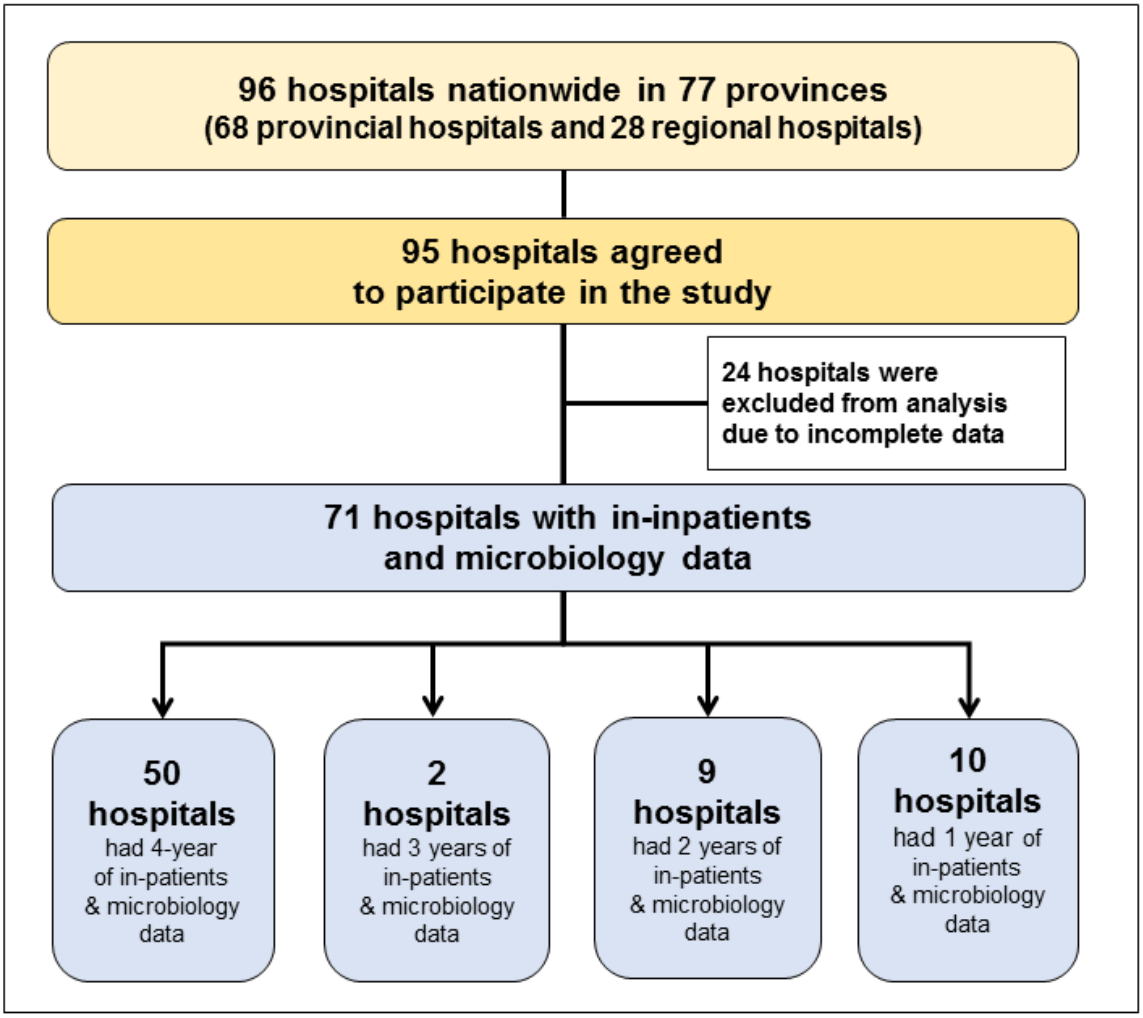

Figure 4-2 Distribution of 95 regional and general hospitals provided hospital data in Thailand.

(a) Map of Thailand. (b) Location of the 95 general and regional hospitals partitipated in the study. Navy blue and green circles represent 71 hospitals and 24 hospitals, of which were included and excluded from the analysis, respectively.

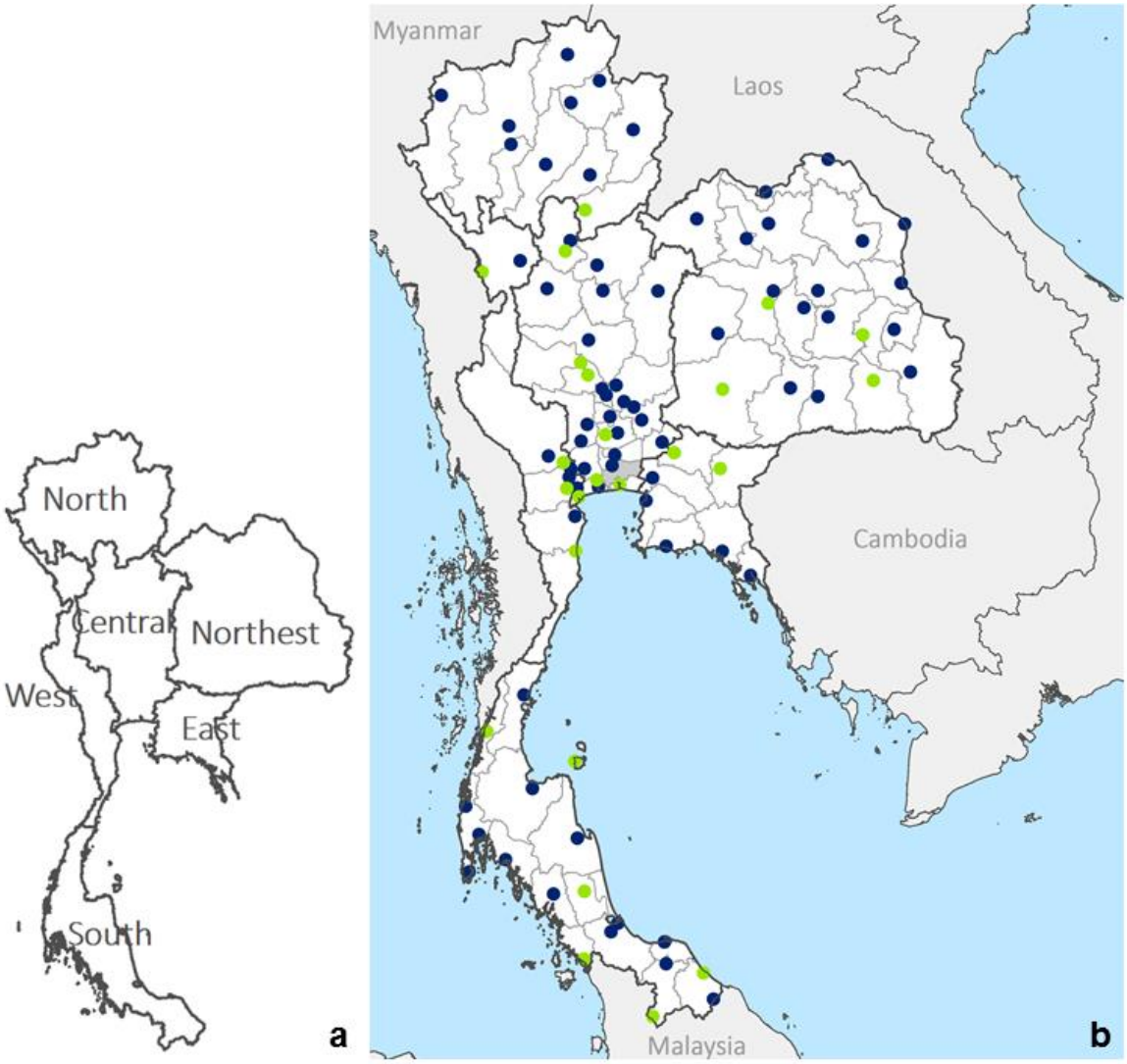

Table 4-2 Incidence and in-hospital mortality of culture-confirmed melioidosis cases diagnosed at 71 hospitals in Thailand from 2012 to 2015

No	Hospital	Province	Region	Year of data included	No. of culture-confirmed melioidosis cases	No. of cases died in the hospital	In-hospital mortality (%)
1	Ang Thong hospital	Angthong	Central	2012	1	1	100%
2	Phanakorn Si Ayutthaya hospital	Ayutthaya	Central	2013,2015	3	1	33%
3	Kamphaeng Phet hospital	Kamphaengphet	Central	2012-2015	66	26	39%
4	Ban Mi hospital	Lopburi	Central	2014-2015	5	3	60%
5	Kingnarai hospital	Lopburi	Central	2012	1	1	100%
6	Nakhon Nayok hospital	Nakhonnayok	Central	2012,2014	24	9	38%
7	Nakhon Pathom hospital	Nakhonpathom	Central	2012-2015	12	2	17%
8	Sawan Pracha Rak hospital	Nakhonsawan	Central	2012-2015	100	42	42%
9	Pranangklao hospital	Nonthaburi	Central	2012-2015	0	0	0%
10	Pathum Thani hospital	Pathumthani	Central	2012	1	1	100%
11	Phetchabun hospital	Phetchabun	Central	2012-2015	53	19	36%
12	Phichit hospital	Phichit	Central	2015	2	1	50%
13	Buddhachinaraj hospital	Phitsanulok	Central	2012-2015	186	61	33%
14	Samut Sakhon hospital	Samutsakhon	Central	2012	1	0	0%
15	Phra Phutthabat hospital	Saraburi	Central	2015	3	2	67%
16	Saraburi hospital	Saraburi	Central	2012-2015	42	12	29%
17	Inburi Hospital	Singburi	Central	2012-2015	0	0	0%
18	Singburi hospital	Singburi	Central	2013	1	1	100%
19	Srisangworn hospital	Sukhothai	Central	2012-2015	10	4	40%
20	Chao Phaya Yommarat hospital	Suphan Buri	Central	2012-2015	22	10	45%
21	Somdejprasangkharach XVII hospital	Suphan Buri	Central	2012,2015	3	0	0%
22	Bhuda Sothon hospital	Chachoengsao	East	2012-2015	113	47	42%
23	Chon Buri hospital	Chonburi	East	2012-2015	107	59	55%
24	Prapokkloa hospital	Chanthaburi	East	2012-2015	68	19	28%
25	Rayong hospital	Rayong	East	2012-2015	76	36	47%
26	Trat hospital	Trat	East	2012-2015	0	0	0%
27	Nakornping hospital	Chiangmai	North	2012,2014	3	0	0%
28	Chiang Rai Prachanukroh hospital	Chiangrai	North	2012-2015	0	0	0%
29	Lampang hospital	Lampang	North	2012-2015	140	24	17%
30	Lamphun hospital	Lamphun	North	2012-2015	0	0	0%
31	Si Sangwan hospital	Maehongson	North	2012-2015	0	0	0%
32	Nan hospital	Nan	North	2012-2015	90	24	27%
33	Chiangkhum hospital	Phayao	North	2012-2015	44	12	27%
34	Phayao hospital	Phayao	North	2012-2015	62	13	21%
35	Phrae hospital	Phrae	North	2014-2015	19	3	16%
36	Amnat Charoen hospital	Amnat Charoen	Northeast	2012	69	8	12%
37	Buengkan Hospital	Buengkan	Northeast	2014-2015	28	9	32%
38	Buri Ram hospital	Buriram	Northeast	2012-2015	372	105	28%
39	Chaiyaphum hospital	Chaiyaphum	Northeast	2012	63	24	38%
40	Kalasin hospital	Kalasin	Northeast	2012-2015	289	79	27%

No	Hospital	Province	Region	Year of data included	No. of culture-confirmed melioidosis cases	No. of cases died in the hospital	In-hospital mortality (%)
41	Khon Kaen hospital	Khon Kaen	Northeast	2012-2015	506	58	11%
42	Loei hospital	Loei	Northeast	2012-2015	61	18	30%
43	Maha Sarakham hospital	Mahasarakham	Northeast	2012-2015	258	54	21%
44	Mukdahan hospital	Mukdahan	Northeast	2012-2015	125	19	15%
45	Nakhon Phanom hospital	Nakhonphanom	Northeast	2012-2015	358	78	22%
46	Nong Bua Lamphu hospital	Nongbualamphu	Northeast	2013-2015	35	6	17%
47	Nong Khai hospital	Nongkhai	Northeast	2012-2015	108	14	13%
48	Roi Et hospital	Roiet	Northeast	2012-2015	496	104	21%
49	Sakonnakhon hospital	Sakonnakhon	Northeast	2012-2015	708	197	28%
50	Sappasitthiprasong hospital	Ubonratchathani	Northeast	2012-2015	1219	234	19%
51	Surin hospital	Surin	Northeast	2012-2015	124	31	25%
52	Udon Thani hospital	Udonthani	Northeast	2012-2015	656	158	24%
53	Chumphonkhetudomsakdi hospital	Chumphon	South	2012-2015	33	13	39%
54	Krabi hospital	Krabi	South	2012-2015	18	3	17%
55	Maharaj Nakhonsithammarat hospital	Nakhonsithammarat	South	2012-2015	58	25	43%
56	Su-ngai Kolok hospital	Narathiwat	South	2012-2015	0	0	0%
57	Pattani hospital	Pattani	South	2014-2015	8	1	13%
58	Phang Nga hospital	Phang Nga	South	2012,2013,	3	1	33%
59	Takuapa hospital	Phang Nga	South	2012, 2014-2015	15	5	33%
60	Vachira Phuket hospital	Phuket	South	2012-2015	79	22	28%
61	Hatyai hospital	Songkhla	South	2012-2015	59	20	34%
62	Songkhla hospital	Songkhla	South	2012-2015	29	11	38%
63	Surat Thani hospital	Suratthani	South	2012-2015	13	2	15%
64	Trang hospital	Trang	South	2012-2015	39	3	8%
65	Yala hospital	Yala	South	2012-2015	20	4	20%
66	Phaholpolpayuhasaena hospital	Kanchanaburi	West	2012-2015	0	0	0%
67	King Mongkut Memorial hospital	Phetchaburi	West	2012-2015	0	0	0%
68	Ban Pong hospital	Ratchaburi	West	2012-2015	0	0	0%
69	Damnoen Saduak hospital	Ratchaburi	West	2012-2015	0	0	0%
70	Photharam hospital	Ratchaburi	West	2015	1	0	0%
71	King Taksinmaharaj Memorial hospital	Tak	West	2012-2015	18	3	17%
				Total	7,126	1,742	24%

Figure 4-3 Incidence rates of culture confirmed melioidosis in Thailand from 2012 to 2015

Provinces are categorized based on incidence rates of culture-confirmed melioidosis observed (dark red, >5 cases per 100,000 population per year; red, >1 to 5 cases per 100,000 population per year; yellow, >0 to 1 case per 100,000 population per year; green, no cases observed; grey, no data obtained).

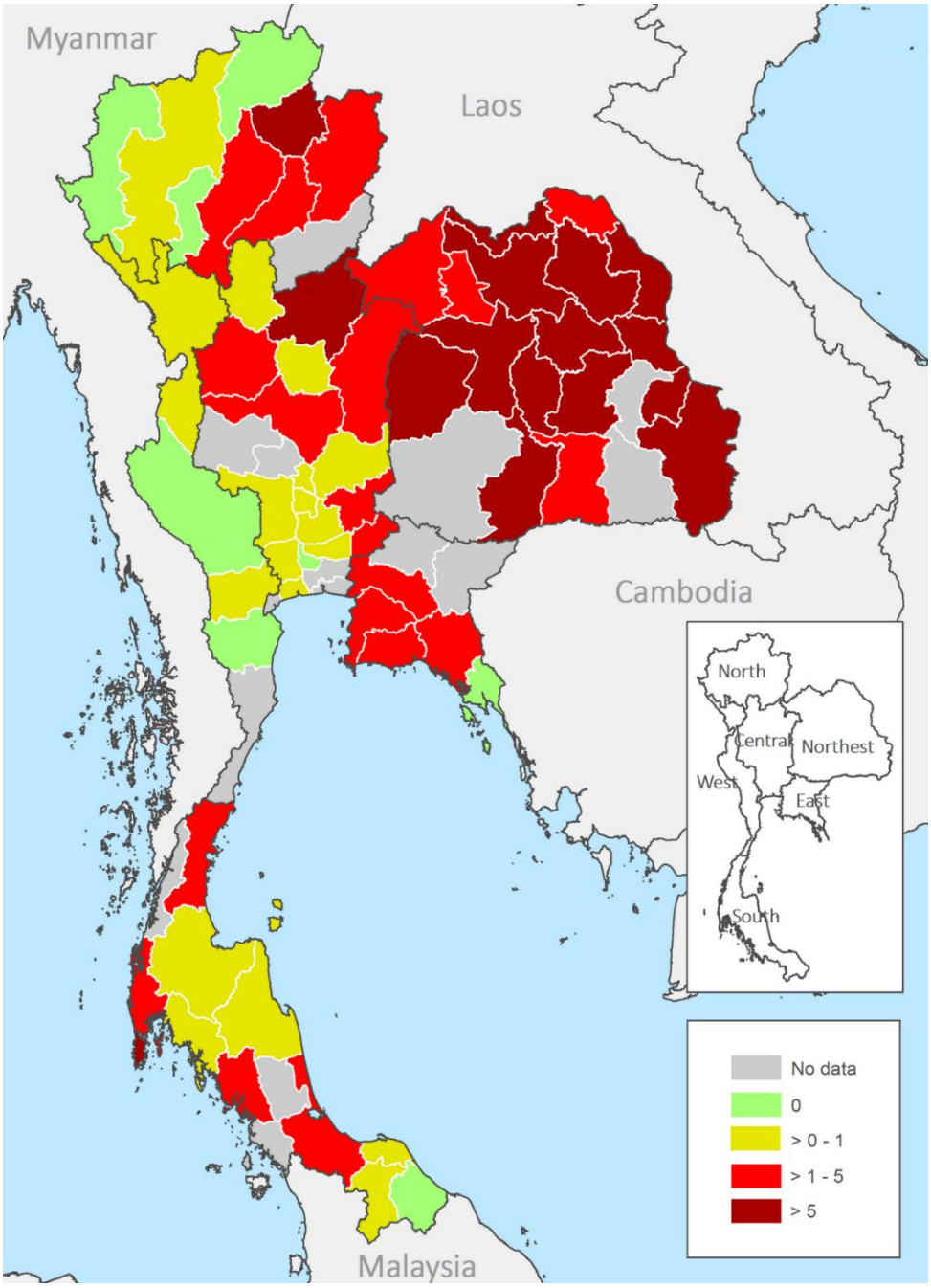

Table 4-3 Incidence rates of culture-confirmed melioidosis from 2012 to 2015 in Thailand, by region

Regions	Number of hospitals	Number of provinces	Melioidosis cases from 2012 to 2015	Total number of population at risk in 2012	Total number of population-year at risk	Incidence rate (per 100,000 population per year)
Central	21	17	536	10,753,504	38,455,625	1.39
East	5	5	364	3,419,147	13,959,934	2.61
North	9	8	358	5,220,558	18,491,803	1.94
Northeast	17	17	5,475	16,139,661	62,745,784	8.73
South	13	11	374	7,348,772	31,143,881	1.20
West	6	4	19	2,679,256	10,756,160	0.18
Overall	**71**	**62**	**7,126**	**45,560,898**	**175,553,187**	**4.06**

Table 4-4 Incidence rates of culture-confirmed melioidosis from 2012 to 2015 in Thailand, by year

Region	Number of hospitals	Numbers of provinces	Number of cases				Incidence rate (per 100,000 population per year)			
			2012[1]	2013[1]	2014[1]	2015[1]	2012[1]	2013[1]	2014[1]	2015[1]
Central	21	17	112	142	155	127	1.04	1.62	1.72	1.28
East	5	5	85	84	113	82	2.49	2.42	3.21	2.31
North	9	8	99	93	85	81	1.90	2.60	1.49	2.03
Northeast	17	17	1332	1359	1481	1303	8.25	8.94	9.45	8.29
South	13	11	97	75	95	107	1.32	1.01	1.16	1.30
West	6	4	10	4	3	2	0.37	0.15	0.11	0.07
Total	**71[1]**	**62[2]**	**1735**	**1757**	**1932**	**1702**	**3.81**	**4.27**	**4.31**	**3.86**

[1]Of 71 hospitals included in study, distribution of four year data were 2012 from 61 hospitals, 2013 from 54 hospitals, 2014 from 58 hospitals and 2015 from 61 hospitals. [2]Eight provinces had the data from more than one hospital, including Lopburi (2), Phayao (2), Ratchaburi (3), Saraburi (2), Singburi (2), Songkhla (2), Phang Nga (2) and Suphanburi (2).

4.4.2 Co-morbidities and presentations of melioidosis

Of 7,126 patients with a first episode of culture-confirmed melioidosis, 4,839 (68%) were male and 2,287 (32%) were female (Table 4-5). Median age was 54 years (IQR 45-63 years, range <1-100 years). The most common co-morbidities identified were diabetes mellitus (43%), followed by hypertension (15%) and chronic kidnesy disease (11%). Culture positivity for *B. pseudomallei* was observed in blood (n=4,910, 69%), sputum (n=1,555, 22%), urine (n=341, 5%), pleural fluid (n=92, 1%), cerebrospinal fluid (n=13, 0.2%) and unidentified pus or fluid (n=1,143, 16%). The most common clinical presentations were bacteraemia (69%) and pneumonia (38%).

Age distribution, number of comorbidities recorded by ICD-10, and clinical presentations differed by region (Table 4-5). The median age of patients was highest in North (57 years) and lowest in West Thailand (48 years) ($p<0.001$). Prevalence of diabetes mellitus was highest in South (48%) and lowest in North Thailand (21%) ($p<0.001$). Bacteraemia was identified highest in East (79%) and lowest in West Thailand (63%). Pneumonia was identified highest in East (46%) and lowest in West Thailand (16%).

4.4.3 Mortality from melioidosis

The 30-day mortality of culture-confirmed melioidosis in 2012 was 40% (696/1,735). 58% (403/696) of deaths occurred in the hospital, while the remainder occurred after hospital discharge with a hospital record of refusal of treatment (76%; 223/293), an improvement in condition (20%; 59/293), transfer to other hospitals (3%; 10/293) or no record of outcome at time of discharge 0.3%; 1/293). Death occurred rapidly, with 285 deaths (41%) occurring within the first two days of admission, 204 (29%) from day 3 to day 7, and the remaining 207 (30%) after 7 days of admission. Among 293 patients who died after hospital discharge, 229 (78%) died within the first

two days of hospital discharge, 28 (10%) from day 3 to day 7, and the remaining 36 (12%) after 7 days of hospital discharge.

The overall mortality rate of culture-confirmed melioidosis in 2012 was 1.5 per 100,000 population. The mortality rate was significantly different by region ($p<0.001$). The mortality rate was highest in Northeast (3.4 per 100,000 population) and lowest in West Thailand (0.04 per 100,000 population).

In the univariable logistic regression models, 30-day mortality was associated with older age, underlying disease of chronic kidney disease, liver disease, malignancy, and presentations with bacteraemia, pneumonia and urinary tract infection (Table 4-6). The underlying diseases of diabetes and thalassemia, and presentations with hepatosplenic abscess and septic arthritis were associated with survival. 30-day mortality was not associated with region ($p=0.14$).

In the final multivariable logistic regression models, 30-day mortality was significantly associated with chronic kidney disease and liver disease as underlying diseases, and with presentations with bacteraemia, pneumonia and urinary tract infection (Table 4-7). Underlying disease of diabetes and presentation with hepatosplenic abscesses were significantly associated with survival outcome.

4.4.4 Comparison between observed data and the Report 506

In 2012, the total number of fatal cases of melioidosis reported to the Report 506 was 13 (Table 4-8). We found that the 71 regional or general hospitals included in the study reported 1,018 melioidosis cases to the Report 506, but reported that only 4 of them died. The other 25 regional or general hospitals not included in the study reported 259 melioidosis cases and that 7 of them died. Primary care units or district hospitals reported another 2,246 melioidosis cases, and that 2 of them died (<0.1%). The discrepancy between the numbers of fatal melioidosis cases observed

and those reported to the Report 506 by the 71 hospitals included in the study in 2012 was more than 100 fold (696 vs. 4).

In 2015, the total number of fatal cases of melioidosis reported to the Report 506 was 111, and 107 of them (96%) were reported from a single regional hospital, Sunpasitthiprasong Hospital, Ubon Ratchathani, in northeast Thailand. Using the hospital admission data, we found that half of those deaths occurred after hospital discharge (51%; 55/107).

4.4.5 Sensitivity analysis

Using data from the year 2012-2015, we evaluated factors associated with in-hospital mortality as an outcome. A temporal trend of in-hospital mortality was not observed ($p=0.98$). In the univariable model, region was associated with in-hospital mortality ($p<0.001$) (Table 4-9).

In a multivariable logistic regression model, in-hospital mortality was significantly associated with an underlying disease of liver disease, and presentations with bacteraemia, pneumonia and urinary tract infection, and region (Table 4-10). The underlying diseases of diabetes and thalassaemia, and presentations with hepatosplenic abscesses, and septic arthritis were negatively associated with in-hospital mortality.

As region was associated with in-hospital mortality, we *ad hoc* evaluated the proportions of patients who died after hospital discharge among those who died within 30 days of hospital admissions in 2012 by region. We found that the proportion was significantly different by region ($p<0.001$); highest in Northeast (50%: 270/541), followed by South (24%; 8/34), North (18%; 6/33), Central (12%; 6/50), East (8%; 3/37) and WestThailand (0%: 0/1).

Table 4-5 Baseline characteristics of 7,126 culture-confirmed melioidosis cases in Thailand from 2012 to 2015

Baseline characteristics	All cases (N=7126)	Northeast (n=5475)	Central (n=536)	East (n=364)	North (n=358)	South (n=374)	West (n=19)	P value
Gender								
• Female	2287 (32.1%)	1788 (32.7%)	152 (28.4%)	125 (34.3%)	108 (30.2%)	107 (28.6%)	7 (36.8%)	0.16
• Male	4839 (67.9%)	3687 (67.3%)	384 (71.6%)	239 (65.7%)	250 (69.8%)	267 (71.4%)	12 (63.2%)	
Age group (years)								
• <1 to 14	264 (3.7%)	219 (4.0%)	13 (2.4%)	12 (3.3%)	4 (1.1%)	16 (4.3%)	0 (0.0%)	<0.001
• 15 to 29	253 (3.6%)	176 (3.2%)	20 (3.7%)	18 (4.9%)	9 (2.5%)	28 (7.5%)	2 (10.5%)	
• 30 to 45	1263 (17.7%)	972 (17.8%)	91 (17.0%)	74 (20.3%)	31 (8.7%)	89 (23.8%)	6 (31.6%)	
• 45 to 64	3874 (54.4%)	3049 (55.7%)	261 (48.7%)	166 (45.6%)	221 (61.7%)	169 (45.2%)	8 (42.1%)	
• >=65	1466 (20.6%)	1053 (19.2%)	151 (28.2%)	94 (25.8%)	93 (26.0%)	72 (19.3%)	3 (15.8%)	
Comorbidities[1]								
• Diabetes mellitus	3045 (42.7%)	2428 (44.3%)	190 (35.4%)	164 (45.1%)	74 (20.7%)	180 (48.1%)	9 (47.4%)	<0.001
• Hypertension	1063 (14.9%)	764 (14.0%)	88 (16.4%)	93 (25.5%)	51 (14.2%)	64 (17.1%)	3 (15.8%)	<0.001
• Chronic kidney disease	815 (11.4%)	688 (12.6%)	35 (6.5%)	38 (10.4%)	27 (7.5%)	26 (7.0%)	1 (5.3%)	<0.001
• Liver disease	661 (9.3%)	513 (9.4%)	49 (9.1%)	55 (15.1%)	17 (4.7%)	27 (7.2%)	0 (0.0%)	<0.001
• COPD	200 (2.8%)	117 (2.1%)	27 (5.0%)	24 (6.6%)	17 (4.7%)	13 (3.5%)	2 (10.5%)	<0.001
• HIV	67 (0.9%)	44 (0.8%)	7 (1.3%)	7 (1.9%)	2 (0.6%)	6 (1.6%)	1 (5.3%)	0.04
• Malignancy	196 (2.8%)	128 (2.3%)	28 (5.2%)	20 (5.5%)	9 (2.5%)	9 (2.4%)	2 (10.5%)	<0.001
• Pulmonary tuberculosis	217 (3.0%)	165 (3.0%)	17 (3.2%)	12 (3.3%)	6 (1.7%)	17 (4.5%)	0 (0.0%)	0.32
• Thalassaemia	151 (2.1%)	117 (2.1%)	10 (1.9%)	6 (1.6%)	7 (2.0%)	11 (2.9%)	0 (0.0%)	0.82
Clinical manifestations[1]								
• Bacteraemia[2]	4910 (68.9%)	3741 (68.3%)	348 (64.9%)	286 (78.6%)	256 (71.5%)	267 (71.4%)	12 (63.2%)	<0.001
• Pneumonia[3]	2705 (38.0%)	2082 (38.0%)	237 (44.2%)	166 (45.6%)	93 (26.0%)	124 (33.2%)	3 (15.8%)	<0.001
• Urinary tract infection[4]	341 (4.8%)	252 (4.6%)	31 (5.8%)	14 (3.8%)	26 (7.3%)	15 (4.0%)	3 (15.8%)	0.03
• Liver/splenic abscess[1]	580 (8.1%)	491 (9.0%)	31 (5.8%)	20 (5.5%)	13 (3.6%)	24 (6.4%)	1 (5.3%)	<0.001
• Septic arthritis[1]	385 (5.4%)	318 (5.8%)	27 (5.0%)	13 (3.6%)	12 (3.4%)	14 (3.7%)	1 (5.3%)	0.10
• Osteomyelitis[1]	63 (0.9%)	40 (0.7%)	6 (1.1%)	3 (0.8%)	3 (0.8%)	9 (2.4%)	2 (10.5%)	<0.001

[1]Comorbidities and clinical manifestations identified by using ICD10 diagnostic codes listed in Table 4-1. [2]Blood culture positive for *B. pseudomallei*. [3]Using ICD10 diagnostic codes incorporated with sputum culture positive for *B. pseudomallei*. [4]Urine culture positive for *B. pseudomallei*

Table 4-6 Factors associated with 30-day mortality in 1,735 culture-confirmed melioidosis cases in 2012

Baseline characteristics	All cases (N=1735)	Died (n=696)	Survived (n=1039)	Crude odds ratio (95% CI)	P value
Gender					
• Female	565 (32.6%)	220 (31.6%)	345 (33.2%)	1.08 (0.87-1.33)	0.49
• Male	1170 (67.4%)	476 (68.4%)	694 (66.8%)		
Age group (years)					
• <1to14	75 (4.3%)	11 (1.6%)	64 (6.2%)	1	<0.001
• 15-29	53 (3.1%)	18 (2.6%)	35 (3.4%)	2.97 (1.25-7.05)	
• 30-45	334 (19.3%)	122 (17.5%)	212 (20.4%)	3.31 (1.67-6.57)	
• 45-64	930 (53.6%)	373 (53.6%)	557 (53.6%)	3.88 (2.01-7.52)	
• 65-115	343 (19.8%)	172 (24.7%)	171 (16.5%)	5.90 (2.99-11.66)	
Comorbidities[1]					
• Diabetes mellitus	717 (41.3%)	243 (34.9%)	474 (45.6%)	0.66 (0.54-0.81)	<0.001
• Hypertension	236 (13.6%)	85 (12.2%)	151 (14.5%)	0.87 (0.65-1.16)	0.34
• Chronic kidney disease	196 (11.3%)	99 (14.2%)	97 (9.3%)	1.73 (1.27-2.36)	<0.001
• COPD	55 (3.2%)	26 (3.7%)	29 (2.8%)	1.45 (0.84-2.51)	0.18
• Pulmonary tuberculosis	59 (3.4%)	28 (4.0%)	31 (3.0%)	1.38 (0.81-2.34)	0.23
• HIV	14 (0.8%)	6 (0.9%)	8 (0.8%)	1.10 (0.38-3.23)	0.89
• Liver disease	138 (8.0%)	82 (11.8%)	56 (5.4%)	2.49 (1.74-3.58)	<0.001
• Malignancy	40 (2.3%)	22 (3.2%)	18 (1.7%)	1.94 (1.02-3.68)	0.04
• Thalassaemia	27 (1.6%)	5 (0.7%)	22 (2.1%)	0.35 (0.13-0.94)	0.04
Clinical manifestations[1]					
• Bacteraemia[2]	1181 (68.1%)	587 (84.3%)	594 (57.2%)	4.12 (3.23-5.26)	<0.001
• Pneumonia[3]	653 (37.6%)	389 (55.9%)	264 (25.4%)	3.98 (3.22-4.92)	<0.001
• Urinary tract infection[4]	104 (6.0%)	63 (9.1%)	41 (3.9%)	2.59 (1.71-3.92)	<0.001
• Liver/splenic abscess[1]	151 (8.7%)	33 (4.7%)	118 (11.4%)	0.38 (0.25-0.57)	<0.001
• Septic arthritis[1]	89 (5.1%)	23 (3.3%)	66 (6.4%)	0.50 (0.30-0.81)	0.005
• Osteomyelitis[1]	13 (0.7%)	2 (0.3%)	11 (1.1%)	0.26 (0.06-1.18)	0.08
Region					
• Northeast	1332 (76.8%)	541 (77.7%)	791 (76.1%)	1	0.32
• Central	112 (6.5%)	50 (7.2%)	62 (6.0%)	1.27 (0.77-2.08)	
• East	85 (4.9%)	37 (5.3%)	48 (4.6%)	1.20 (0.68-2.11)	
• North	99 (5.7%)	33 (4.7%)	66 (6.4%)	0.83 (0.47-1.45)	
• South	97 (5.6%)	34 (4.9%)	63 (6.1%)	0.86 (0.51-1.44)	
• West	10 (0.6%)	1 (0.1%)	9 (0.9%)	0.17 (0.02-1.52)	

[1]Comorbidities and clinical manifestations identified by using ICD10 diagnostic codes listed in Table 4-1. [2]Blood culture positive for *B. pseudomallei*. [3]Using ICD10 diagnostic codes incorporated with sputum culture positive for *B. pseudomallei*. [4]Urine culture positive for *B. pseudomallei.*

Table 4-7 Factors associated with 30-day mortality by a multivariable logistic regression model stratified by hospitals

Baseline characteristics	Adjusted odds ratio (95%CI)	P value
Gender (male)	0.76 (0.60-0.98)	0.03
Age (years)	1.01 (1.01-1.02)	<0.001
Comorbidities[1]		
• Liver disease	2.55 (1.69-3.85)	<0.001
• Chronic kidney disease	1.74 (1.22-2.49)	0.002
• Diabetes mellitus	0.49 (0.38-0.63)	<0.001
Clinical manifestations[1]		
• Bacteraemia[2]	6.96 (5.22-9.28)	<0.001
• Pneumonia[3]	5.83 (4.53-7.52)	<0.001
• Urinary tract infection[4]	3.56 (2.15-5.88)	<0.001
• Liver/splenic abscess[1]	0.46 (0.29-0.73)	0.001

[1]Comorbidities and clinical manifestations identified by using ICD10 diagnostic codes listed in Table 4-1. [2]Blood culture positive for *B. pseudomallei*. [3]Using ICD10 diagnostic codes incorporated with sputum culture positive for *B. pseudomallei*. [4]Urine culture positive for
B. pseudomallei.

Table 4-8 Incidences and mortalities of melioidosis cases diagnosed by microbiology laboratories in regional or general hospitals compared with those officially reported to the Report 506 from 2012 to 2015

Year	Type of hospital	Culture-confirmed melioidosis[1]		Report 506[2]	
		Number of cases[1]	Number of fatal cases[1]	Number of cases[3]	Number of fatal cases[3]
2012	Primary care	Not availble	Not availble	2426	2
	Regional or general hospital	1,735	696	1,277 (1,018)[3]	11 (4)[3]
2013	Primary care	Not availble	Not availble	1,821	0
	Regional or general hospital	1,757	459	1,009 (799)[3]	4 (3)[3]
2014	Primary care	Not availble	Not availble	1,677	3
	Regional or general hospital	1,932	482	869 (695)[3]	9 (8)[3]
2015	Primary care	Not availble	Not availble	2,042	1
	Regional or general hospital	1,702	382	1,184 (967)[3]	111 (111)[3]
	Total	**7,126**	**1,742**	**12,305 (3,479)[3]**	**141 (126)[3]**

[1]From 71 hospitals participating in the study (data of this study). Fatal cases in 2012 were based on 30-day mortality, while fatal cases in 2013, 2014 and 2015 were based on in-hospital mortality. [2]Reported melioidosis are either probable cases or confirmed cases of melioidosis. Probable cases defined as clinically compatible illness and IHA titer ≥1:160 or IFA >1:400. Confirmed melioidosis defined as clinically compatible illness and IHA titer ≥1:160 or IFA >1:400 or culture positive for *B. pseudomallei*. [3]Numbers of cases and deaths in parenthesis were from participating general and regional hospitals in this study.

Table 4-9 Factors associated with in-hospital mortality

Baseline characteristics	All cases (N=7,126)	In-hospital mortality (n=1,742)	Discharged with survival outcomes (n=5384)	Crude odds ratio (95%CI)	P value
Gender					
• Female	2287 (32.1%)	577 (33.1%)	1710 (31.8%)	0.94 (0.83-1.05)	0.27
• Male	4839 (67.9%)	1165 (66.9%)	3674 (68.2%)		
Age group (years)					
• <1 to 14	264 (3.7%)	24 (1.4%)	240 (4.5%)	1	<0.001
• 15 to 29	253 (3.6%)	54 (3.1%)	199 (3.7%)	2.70 (1.60-4.55)	
• 30 to 45	1263 (17.7%)	309 (17.7%)	954 (17.7%)	3.34 (2.14-5.20)	
• 45 to 64	3874 (54.4%)	946 (54.3%)	2928 (54.4%)	3.48 (2.26-5.36)	
• >=65	1466 (20.6%)	409 (23.5%)	1057 (19.6%)	4.03 (2.59-6.26)	
Comorbidities[1]					
• Diabetes mellitus	3045 (42.7%)	654 (37.5%)	2391 (44.4%)	0.74 (0.66-0.84)	<0.001
• Hypertension	1063 (14.9%)	256 (14.7%)	807 (15.0%)	0.92 (0.78-1.07)	0.28
• Chronic kidney disease	815 (11.4%)	212 (12.2%)	603 (11.2%)	1.13 (0.95-1.34)	0.17
• COPD	200 (2.8%)	64 (3.7%)	136 (2.5%)	1.35 (0.99-1.84)	0.06
• Pulmonary tuberculosis	217 (3.0%)	62 (3.6%)	155 (2.9%)	1.22 (0.90-1.66)	0.20
• HIV	67 (0.9%)	19 (1.1%)	48 (0.9%)	1.15 (0.67-1.99)	0.61
• Liver disease	661 (9.3%)	231 (13.3%)	430 (8.0%)	1.75 (1.47-2.08)	<0.001
• Malignancy	196 (2.8%)	42 (2.4%)	154 (2.9%)	0.80 (0.56-1.14)	0.22
• Thalassaemia	151 (2.1%)	20 (1.1%)	131 (2.4%)	0.46 (0.29-0.75)	0.002
Clinical manifestations[1]					
• Bacteraemia[2]	4910 (68.9%)	1478 (84.8%)	3432 (63.7%)	3.40 (2.93-3.94)	<0.001
• Pneumonia[3]	2705 (38.0%)	1046 (60.0%)	1659 (30.8%)	3.60 (3.20-4.04)	<0.001
• Liver/splenic abscess[1]	580 (8.1%)	52 (3.0%)	528 (9.8%)	0.29 (0.22-0.39)	<0.001
• Septic arthritis[1]	385 (5.4%)	52 (3.0%)	333 (6.2%)	0.48 (0.35-0.65)	<0.001
• Urinary tract infection[4]	341 (4.8%)	137 (7.9%)	204 (3.8%)	2.18 (1.73-2.74)	<0.001
• Osteomyelitis[1]	63 (0.9%)	4 (0.2%)	59 (1.1%)	0.21 (0.07-0.58)	0.003
Regions					
• Northeast	5475 (76.8%)	1196 (68.7%)	4279 (79.5%)	1	<0.001
• Central	536 (7.5%)	196 (11.3%)	340 (6.3%)	2.15 (1.55-2.99)	
• East	364 (5.1%)	161 (9.2%)	203 (3.8%)	2.75 (1.84-4.12)	
• North	358 (5.0%)	76 (4.4%)	282 (5.2%)	0.97 (0.64-1.47)	
• South	374 (5.2%)	110 (6.3%)	264 (4.9%)	1.41 (0.99-1.99)	
• West	19 (0.3%)	3 (0.2%)	16 (0.3%)	0.65 (0.16-2.56)	

[1] Comorbidities and clinical manifestations identified by using ICD10 diagnostic codes listed in Table 4-1. **[2]** Blood culture positive for *B. pseudomallei*. **[3]** Using ICD10 diagnostic codes incorporated with sputum culture positive for *B. pseudomallei*. **[4]** Urine culture positive for *B. pseudomallei*.

Table 4-10 Factors associated with in-hospital mortality by a multivariable logistic regression model stratified by hospital

Baseline characteristics	Adjusted odds ratio (95%CI)	P value
Gender (male)	0.82 (0.72-0.93)	0.002
Age group (years)		
• <1 to 14	1	0.02
• 15 to 29	1.91 (1.09-3.36)	
• 30 to 45	2.16 (1.34-3.50)	
• 45 to 64	2.06 (1.29-3.30)	
• >=65	2.04 (1.27-3.28)	
Comorbidities[1]		
• Diabetes mellitus	0.67 (0.59-0.76)	<0.001
• Liver disease	1.48 (1.22-1.79)	<0.001
• Thalassaemia	0.54 (0.32-0.91)	0.02
Clinical manifestations[1]		
• Bacteraemia[2]	3.99 (3.41-4.67)	<0.001
• Pneumonia[3]	3.71 (3.28-4.20)	<0.001
• Urinary tract infection[4]	2.35 (1.82-3.04)	<0.001
• Septic arthritis[1]	0.71 (0.52-0.98)	0.04
• Liver/splenic abscess[1]	0.36 (0.26-0.49)	<0.001
• Osteomyelitis[1]	0.40 (0.14-1.18)	0.10
Regions		
• Northeast	1	<0.001
• Central	2.26 (1.56-3.29)	
• East	2.52 (1.60-3.98)	
• North	0.93 (0.58-1.48)	
• South	1.54 (1.04-2.28)	
• West	0.92 (0.21-3.98)	

[1]Comorbidities and clinical manifestations identified by using ICD10 diagnostic codes listed in Table 4-1. [2]Blood culture positive for *B. pseudomallei*. [3]Using ICD10 diagnostic codes incorporated with sputum culture positive for *B. pseudomallei*. [4]Urine culture positive for *B. pseudomallei*.

4.5 Discussion

Our findings affirm that melioidosis is endemic and an important cause of death in Thailand. *B. pseudomallei* is commonly identified by clinical microbiology laboratories in most regional or general hospitals in Thailand. The 30-day mortality of those culture-confirmed melioidosis cases is high at 40%. Our findings show that regional and general hospitals rarely report the cases or their outcomes through the Report 506 of MoPH. This all suggests that data from the national disease surveillance system in resource limited settings (such as Report 506) should not be used alone to derive the burden of diseases and prioritize actions, and that an improvement in the reporting of melioidosis cases and their outcomes in Thailand is critically needed. Given the high burden of melioidosis observed countrywide, policy makers in Thailand should also raise the priority afforded to the disease, and consider implementing a large national campaign to raise awareness and implement prevention measures.

Our study systematically estimated incidence rates of culture-confirmed melioidosis cases in all six regions in Thailand. The high incidence rates observed in East, North, Central and South Thailand was supported by multiple further sources of evidence presented in the latest review conducted by the Thailand Melioidosis Network (Hinjoy et al., 2018). Our observed incidence of melioidosis in the East is lower than that estimated by an active population-based surveillance study (Bhengsri et al., 2011b); 2.6 vs. 4.9 per 100,000 population per year, respectively. This is probably because data from Sa Kaeo province was not available for our study, and that our study was based on passive surveillance of cases diagnosed by routine microbiology laboratories, while Bhengsri et al's study implemented active blood culture surveillance during their study period. Also the incidence rate of melioidosis in the Northeast estimated in this study is lower than that reported in a retrospective study conducted in Ubon Ratchathani; 8.7 vs. 12.7 per 100,000

population per year (Limmathurotsakul et al., 2010b). This was probably because our data was the average of the 20 provinces in Northeast Thailand, while Ubon Ratchathani is equipped with a research centre based in the regional hospital focusing on diagnosis of melioidosis. Therefore, our observed incidence rates should be considered as a minimum incidence rate based on by routine microbiology reports, and the true incidence rate could be higher.

Culture confirmed melioidosis cases were not found in eight provinces participating in our study; three from the North, two from the West Thailand, and one each from the East, Central and South. However, high incidences of culture-confirmed melioidosis cases are observed in their bordering provinces. It is possible that *B. pseudomallei* may be actively misidenitified as *Pseudomomas* species, other *Burkholderia* species or contaminants in laboratories in those eight provinces (Podin et al., 2013). This suggests that microbiological training should be provided to the microbiological laboratories, and guidelines for the diagnosis and treatment of melioidosis issued to the physicians in those provinces.

Our study suggests that clinical presentations of melioidosis may differ by region. Bacteraemia was the most common clinical presentation (69%), highest in East (72%) and lowest in West Thailand (63%). Those percentages are higher than the proportion of bacteraemia observed in Ubon Ratchathani (55%) (Limmathurotsakul et al., 2010a) and in Darwin, Australia (55%) (Currie et al., 2010) in research centres where selective media is routinely used for all non-sterile specimens. Bacteraemia is also associated with routes of infection (Lim et al., 2016, Wiersinga et al., 2018); inhalation and digestion routes are associated with pneumonia and bacteraemia, respectively. Therefore, the difference of bacteraemia and other clinical presentations including pneumonia and urinary tract infection could be due to different characteristics of the baseline populations, different risk of exposure due to different occupations based on geographical

variations, and different practices of physicians and clinical microbiological laboratories in the region. Variations in the patterns of clinical presentations and co-mordibidities identified could also be due to different practices of converting the final diagnoses into ICD-10 codes by attending physicians in each region.

The high 30-day mortality and rapid death of melioidosis patients in all regions in Thailand is concerning. Our observed 30-day mortality (40%) is comparable with those previously reported from a large cohort in Thailand; including 36% in Sa Kaeo, East Thailand, and Nakhon Panom, Northeast Thailand (Bhengsri et al., 2011b) and 43% in Ubon Ratchathani, Northeast Thailand (Limmathurotsakul et al., 2010b). This could be because the recommended antimicrobials for acute melioidosis, including ceftzidime or meropenem, are widely available in Thailand, and all Thai citizens are able to seek health care without charges as they are covered by the universal coverage. The possible contributing factors to high mortality in Thailand compared to the lower mortality in Australia (14%) (Currie et al., 2010) may include lower levels of supportive care and intensive care facilities, differences in the infecting organisms, and relatively delayed diagnosis and administration of effective antibiotics.

The mortality of melioidosis is severely underreported by the national surveillance system (Report 506). There are a few explanations of the low number of fatal melioidosis cases reported via the Report 506 system. First, the general definition of melioidosis used in the Report 506 system, in which melioidosis could be diagnosed in patients who have relevant clinical symptoms and IHA titer ≥1:160. More than sixty percent of melioidosis cases were reported from PCUs or district hospitals which do not have a microbiology laboratory to confirm the diagnosis of melioidosis. Therefore, most melioidosis cases reported to the Report 506 system probably are misdiagnosed cases by IHA. Second, there is a failure in general and regional hospitals to report

cases and the final outcomes of culture-confirmed melioidosis cases. Laboratory isolation and identification of *B. pseudomallei* can take up to 7 days; 40% of culture-confirmed cases die within 3 days of admission or could be discharged, in both cases prior to the final diagnosis making it into the medical records. A proportion of cases may have been properly diagnosed while the patients are still in hospitals. However, healthcare workers may not report the cases, or report the case but not confirm and report the final outcome to the BOE, MoPH, Thailand (Hinjoy et al., 2018).

The limitations of this study are that private hospitals, specialized hospitals such as military hospitals and psychiatric hospitals, hospitals in Bangkok, and university hospitals were not included in the study. It is possible that participating hospitals may still misidentify a proportion of the *B. pseudomallei* isolates, and selective media for *B. pseudomallei* is not used for non-sterile clinical specimens such as sputum. Data of National Death Registry in 2013-2015 was not available. Hence, incidence of and deaths from melioidosis in this study are likely to be only a proportion of the true burden.

To date, this study is the largest population-based study carried out in Thailand. The study emphasizes that melioidosis is an important cause of death throughout Thailand. With high incidence rates and high 30-day mortality, the disease poses a great health burden to people and health care providers in the country. This study also demonstrates that data from national notifiable disease-surveillance systems in resource-limited settings may need to be verified and supplemented retrospectively by integrating information from readily available databases. Potential improvements to the national notifiable disease-surveillance data on melioidosis are being devised and reported to policy makers.

Chapter 5: General discussion and conclusions

This thesis describes studies performed between 2013-2017 on three important topics: (1) distribution of environmental *B. pseudomallei* and its associated environmental factors in Thailand; (2) association between IHA positivity and exposure to *B. pseudomallei* and *B. thailandensis* in healthy people; and (3) the distribution and burden of human melioidosis in Thailand.

The environmental survey in East, Central and Northeast Thailand defines the risk areas of melioidosis in all three regions. *B. pseudomallei* is widely spread in the East and Northeast, and unevenly distributed in Central Thailand. The study also demonstrates that the pathogen is more commonly found in soil with lower levels of organic matter and nutrients, suggesting that agriculture practices resulting in decline in soil nutrients may impact the presence and amount of *B. pseudomallei* in affected areas. The study is also the first to report the presence of a variant of *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide (BTCV) in the environment in Thailand. The study on serological response against *B. pseudomallei* in healthy rice farmers shows that IHA seropositivity of the farmers in Thailand is associated with presence of *B. pseudomallei* rather than presence of *B. thailandensis* or BTCV. This supports the utility of IHA in evaluating exposure to *B. pseudomallei*, and suggest that exposure to *B. thailandensis* and BTCV in the environment is not the main factor associated with the IHA seropositivity in healthy individuals.

The extensive descriptive retrospective study affirms the high burden of melioidosis in Thailand and highlights the fact that melioidosis is diagnosed regularly in large hospitals with microbiology facilities not only in the whole northeast Thailand, but also in Central, East and

South Thailand. Furthermore, the study also describes limitations in the national surveillance system of Thailand (Report 506) and provides suggested strategies to improve the surveillance of melioidosis in Thailand.

This chapter discusses key findings of this thesis and elaborates on possible future directions for research.

5.1 Distribution of environmental *B. pseudomallei* and risk of melioidosis

The overall prevalence of environmental *B. pseudomallei* in rice fields in the East, Central and Northeast are higher than the hypothesized prevalence. Our findings provide the geographical setting for preventive measures as well as raising awareness of this disease among healthcare workers in affected areas. Our data shows a high prevalence of environmental *B. pseudomallei* in six provinces in the East (57%), suggesting a high risk of infection to people who are living in this region (Chapter 2). Our results in Chapter 4 confirm this suggestion as culture confirmed melioidosis was commonly found in almost every province in East Thailand. We conclude that East Thailand is another highly endemic region for melioidosis in Thailand. In contrast to the East, in the Central region *B. pseudomallei* was isolated from three of seven provinces, and the positivity rate was significantly lower than in the East and Northeast (Chapter 2). We also found that those three provinces (Phitsanulok, Phetchabun, and Nakhon Nayok) with presence of *B. pseudomallei* in the soil had a high incidence of melioidosis in 2012-2015 (Chapter 4). However, three provinces in which the soil samples were culture negative for the organism (Lopburi, Saraburi, and Pathum Thani) also had high incidence of melioidosis. Similar discrepancies between findings from the soil survey and the hospital data study are observed in two provinces in the Northeast (Loei and Chaiyaphum). This may be because our sample size for the environmental study was calculated specifically for determining associated factors with presence of the organism; hence, the sample

size was not large enough for defining a risk map in all provinces included in the study. Another explanation is that the bacterial load of *B. pseudomallei* in negative rice fields in Central Thailand might be lower than the bacterial culture detection limit. In order to define a risk map of melioidosis, sample size calculations should correspond with the aims of defining a risk map and should proportionally correspond to size of geographical areas of the study. In addition, the wide distribution of *B. thailandensis* and BTCV in the environment (Chapter 2) could make differentiation of *B. pseudomallei* from *B. thailandensis* and BTCV in environmental samples difficult. BTCV possess *B. pseudomallei*-like CPS, hence using identification methods which are specific for CPS alone may give false positives for *B. pseudomallei*. To ensure accurate identification, biochemistry profile for arabinose assimilation or other methods such as PCR assay or MALDI-TOF should be used in combination.

Movement of humans and animals within the country is common (Limmathurotsakul et al., 2012a). Previously it was unclear whether melioidosis cases found in what were previously considered non-endemic regions (such as Central, East, North, South and West Thailand) were imported from the northeast or originated in the regions. Our enviromental findings suggested that a proportion of human and animal melioidosis observed in both Central and East Thailand were locally infected cases.

Further defining the distribution of environmental *B. pseudomallei* is important for the development of a risk map for melioidosis, since this will inform the geographical areas that will benefit from preventive measures, as well as raising awareness of this disease among healthcare workers in affected areas. We observed culture-confirmed meliodosis in all provinces in Central, North, South, and West Thailand (Chapter 4), where there is still limited information on the presence of environmental *B. pseudomallei*. More environmental sampling studies are indicated to

define the risk areas. We also recommend that public health officials should conduct soil or water surveys alongside their case surveillance investigations. This will help expand the risk map gradually and efficiently over time, and result in better diagnosis, treatment, prevention and control of melioidosis in the country.

5.2 Factors associated with presence of *B. pseudomallei*

Conducting an environmental survey and evaluating soil characteristics simultaneously in this study expands our understanding of factors associated with the presence of *B. pseudomallei* in rice fields and explains the presence or absence of *B. pseudomallei* in different regions outside of Northeast Thailand. This is important because previous published findings on factors associated with the presence of the organism in the natural setting are not consistent.

The presence of *B. pseudomallei* is associated with nutrient-depleted soil in rice fields in Thailand (Chapter 2). Because nutrient levels in the soil are largely affected and effected by agricultural practices, this finding implies that agricultural practice may also affect the burden of *B. pseudomallei* in the soil. Further investigations are required to evaluate whether changes in agricultural practices could effectively enhance soil nutrients, and whether these could reduce the distribution of *B. pseudomallei* in rice fields.

Soil organic matter was negatively associated with the presence of *B. pseudomallei*, and that is consistent with two previous environmental studies in Northern Australia (Baker et al., 2015) and Northeast Thailand (Ngamsang et al., 2015). Organic matter contains vital nutrients and influences the diversity and biological activity of soil organisms (FAO, 2005). It could be speculated that soil physicochemical properties may reflect another causal determinant, which is competition among soil microorganisms. This is supported by an environmental study showing that low microbial density in soil is associated with the presence of *B. pseudomallei* (Sermswan et

al., 2015, Potisap et al., 2018) and that *Bacillus amyloliquefaciens* extracted from soil samples can inhibit the growth of *B. pseudomallei* (Potisap et al., 2018).

We propose that evaluating the diversity of soil microorganisms and soil physicochemical properties simultaneously in other regions and worldwide will further expand our understanding of factors associated with presence of *B. pseudomallei*. Conducting longitudinal soil sampling studies in a natural experiment would also inform causal relationship between soil characteristics and the presence of the organism.

In addition, co-localization of *B. thailandensis* and *B. pseudomallei* in the same rice field is not uncommon (Chapter 3), which is in line with a previous environmental study in Khon Kaen, northeast Thailand (Sermswan et al., 2015). This finding differs from previous studies that report that *B. pseudomallei* inhibited growth of *B. thailandensis*. Any inhibitory effect of *B. pseudomallei* on *B. thailandensis* may influence a lower prevalence of co-localization between the two species. However, both species could be isolated from the same soil samples in this study. This could be because the two species rarely have direct contact in the real soil environment. Our finding also suggests that the presence of *B. thailandensis* may not be associated with either the presence or absence of *B. pseudomallei* in the environment.

5.3 Presence of *B. thailandensis* and its implications for seropositivity in humans who are exposed to the organism in environment

B. thailandensis is commonly isolated in all three regions (Northeast, East and Central Thailand), while BTCV is less common but associated with *B. thailandensis* (Chapter 3). Our results suggest that exposure to environmental *B. thailandensis* and BTCV makes a limited contribution to IHA seropositivity in farmers. This supports the recommendation that IHA could

be used to measure exposure to environmental *B. pseudomallei* (Hoffmaster et al., 2015), even in areas containing other closely related *Burkholderia* species.

Our study is the first report of BTCV in Thailand, and a high prevalence of BTCV is observed in East Thailand. In a mouse model, inoculation of BTCV produced protective immunity to melioidosis and may be a candidate for an attenuated vaccine for melioidosis (Scott et al., 2013). Whether live BTCV has a potential to be a vaccine for melioidosis either in humans or animals should be investigated in future. Nonetheless, our study suggests that routine exposure to BTCV in the environment may have only limited impact on the increase in the crude seroresponse against *B. pseudomallei*.

5.4 Burden of human melioidosis in Thailand

Our findings define the distribution of cultured-confirmed melioidosis nationwide, and confirms that melioidosis poses a great threat to public health (Chapter 4). With the evidence derived from Chapter 2 (presence of *B. pseudomallei*) to Chapter 4, this supports an expansion of areas considered endemic for melioidosis from the Northeast to the whole country, including North, East, Central, West and South Thailand. Our data did not find culture-confirmed melioidosis in eight provinces; however, this finding did not indicate the absence of risk for melioidosis in those provinces. This is because a number of culture-confirmed melioidosis cases were observed in adjacent provinces. The absence of melioidosis cases in these eight provinces likely indicates misdiagnosis of melioidosis and misidentification of *B. pseudomallei* in the hospitals due to lack of awareness of the disease and of experience in identification of the organism amongst physicians and laboratory staff.

Diabetes mellitus and older age are the main predisposing factors observed in patients from all regions. Changes in the age profile of the population in Thailand toward an aging society and

increase in the incidence of diabetes mellitus are expanding the size of the population susceptible to *B. pseudomallei* infection. This suggests that the true incidence of melioidosis may increase in Thailand in the future. Improving the awareness of melioidosis in the whole population throughout the country would be the fastest and most cost-effective disease prevention and control strategy. In addition, the availability of new effective antimicrobials and vaccines are not foreseen in the near future. The problem of the high 30-day mortality of about 40% in Thailand has not been resolved. This high mortality has been observed for more than 30 years, largely unchanging since the change of parenteral antibiotic to ceftazidime influenced by landmark clinical trial in 1989 which reduced the mortality from about 80% to 40%. Our findings suggests the necessity of increasing awareness and strengthening capacity in the diagnosis, treatment and prevention of melioidosis nationwide. These could all in themselves lead to an improvement in disease outcomes. The defined distribution of human melioidosis derived from our surveillance study could be used to prioritize resource allocations for training programs for both clinicians and laboratory staff.

The rapid mortality of melioidosis countrywide, including in the Northeast Thailand where awareness of disease is high, may be attributed to a few factors. Although our data were from hospitals equipped with microbiology facilities, delay in diagnosis or in providing effective treatment could not be ruled out. Communication among attending physicians, collecting adequate and a wide range of clinical specimens, and reporting the culture results in a timely fashion are still problems observed in many large hospitals countrywide. The other possible contributing factor includes a delay in seeking medical care. Patients who present to hospital with fulminant disease often have high mortality despite rapid effective treatment.

Underreporting cases of notifiable diseases is a common pitfall of passive disease surveillance worldwide. This is because reporting cases is an additional burden to the current already overloaded health care system. Likewise, reporting melioidosis in Thailand requires hospital staff to complete separate Report 506 forms, and submit these to the surveillance system. Thus, the incidence of melioidosis reported by the surveillance system depends on the rate of completion of Report 506 forms. Form completion rates could vary widely amongst hospitals and regions. For example, the hospital that has a dedicated surveillance team is likely to report more cases than hospitals that do not have resources for reporting.

Based on our findings, several recommendations could be made to improve surveillance of melioidosis more effectively. First, the national notifiable disease surveillance system and policy makers should utilize all laboratory and hospital data from all hospitals in the country when determining the incidence and mortality of culture-confirmed melioidiosis and other notifiable diseases, as we have performed in this study. They should retrospectively verify and supplement the data of the national notifiable disease-surveillance system with the observed laboratory-confirmed cases. Second, we propose that the case definition of melioidosis for reporting to surveillance system be changed to culture-confirmed melioidosis only. This will increase specificity of reported cases and reduce the resources required for reporting at every level. The accurate number of culture-confirmed cases and their mortality outcomes would be more helpful than cases innacurately misdiagnosed as melioidosis based on serological assays. Third, policy makers should initiate the development of standard operating procedures for *B. pseudoamllei* identification (for microbiological laboratories) and guidelines for diagnosis, treatment and prevention (for healthcare workers). In addition, policy makers should provide regular formal training to microbiology laboratories and healthcare workers nationwide. Lastly, policy makers

need to raise awareness of melioidosis by disseminating to both the public and and to healthcare workers key information about meliodosis such as its clinical manifestations, diagnosis (including identification of *B. pseudomallei*), and prevention. Appendix 2 is an example of an initiative to raise awareness of melioidosis among the public and health care workers in Thailand. The pamphlet contains information on melioidosis, and was developed and published in July 2018 under a collaboration between Department of Disease Control, BOE, MoPH and Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Thailand. This pamphlet will be distributed to hospitals nationwide in Thailand by BOE, MoPH, Thailand.

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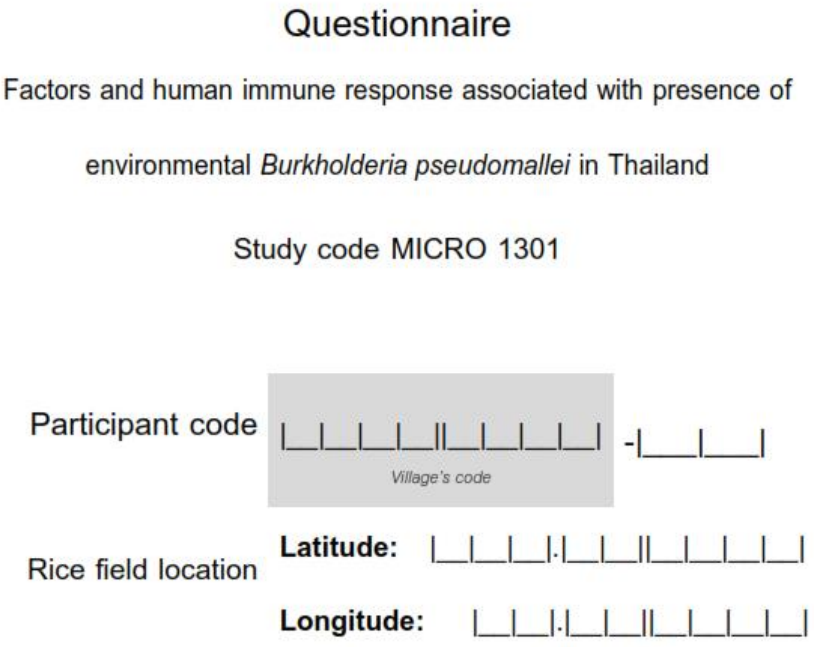
Appendices

Appendix 1. Interviewee-based questionaire to collect information of farmers' demographics, sampled rice field and agricultural practice



Questionnaire

Factors and human immune response associated with presence of environmental *Burkholderia pseudomallei* in Thailand

Study code MICRO 1301

Participant code |__|__|__|__||__|__|__|__| -|___|___|

Village's code

Rice field location **Latitude:** |__|__|__|.|__|__||__|__|__|__|

Longitude: |__|__|.|__|__||__|__|__|__|

Micro1301 version 3.0, 3 January 2014 Page 1 / 6

(Based on Questionnaire Thai version 3.0, 3 January 2014)

participant code | *Village's code* --|___|___|

Completion guideline

This questionnaire contains 3 main sections.

Section A:	**Information of related activities to the sampled rice field** To understand characteristics and exposure activities to the sampled rice field within past 12-month from sampling date
Section B:	**Your information** To be used as baseline information and to understand associated factors related to immune response to the melioid-bacteria in your rice field (if found)
Section C:	**Rice field information (only for rice field owner)** To be used as a baseline information and to understand associated factors related to presence of melioid-bacteria in your rice field (if found)

Wording definition:

- "This rice field" is defined as the rice field which is chosen for this research study and for soil collection.

Questionnaire completion:

- Please read the questions and make a mark in answer box which best represents the true information by ticking in the box such as ☑ or ☒
- If none of the available answers match with your answers please specify in the bracket "……"

- **This questionnaire is designed for self-completion under the study staff's assistances.**
- **In case of illiterate participant, study staff will read questions and help completing the questionnaire for you.**

participant code | *Village's code* | --|___|___|

Section A: **Information of related activities to the sampled rice field**

To understand characteristics and exposure activities to the sampled rice field within past 12-month from sampling date

A1 **You have been exposed to this rice field because**

[1] ☐ I own this rice field.

[2] ☐ I am a family member of rice field owner i.e. husband, wife, children, relatives, etc.

[3] ☐ I rent this rice field.

[4] ☐ I am a rice framing worker.

[5] ☐ I am a neighbour and have been asked to work at this rice field.

[6] ☐ Others, please specify……

A2 **When was the last time you have been exposed to this rice field**

[1] ☐ In last 1-3 months

[2] ☐ Longer than 3 months but not longer than 6 months

[3] ☐ Longer than 6 months but not longer than 12 months

[4] ☐ Longer than 12 months

[5] ☐ I cannot remember.

Section B: **Your information**

To be used as a baseline information and to understand associated factors related to immune response to the melioid-bacteria in your rice field (if found)

B1 **Gender** [1] ☐ Male [2] ☐ Female

B2 **Age: please specify year of birth or age**

Year of birth |___|___|___|___| **or Age** |___|___| **years**

e.g. |*1*|*9*|*7*|*7*| |*3*|*6*|*years*

B3 **Have you been living in this village since you were born?**

[1] ☐ Yes

[2] ☐ No. Please specify your hometown; District……Province……

B4 **In the past 12-month, have you ever lived in other province apart from current address?**

[1] ☐ No (Please skip to question B5)

[2] ☐ Yes. Please specify province ……⇨Please answer question B4.1

B4.1 During your stay in another province, have you ever been exposed to soil or water?

[1] ☐ No

[2] ☐ Yes. Please specify exposure activities:.....

B5 Have you been diagnosed with the following diseases?

5.1) Diabetes	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.2) Hypertension	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.3) Chronic renal failure	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.4) Cancer	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.5) Asthma	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.6) Chronic obstructive pulmonary disease (COPD)	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.7) Tuberculosis	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.8) Thalassemia	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know
5.9) Systemic Lupus Erythematosus (SLE)	[1] ☐ Yes	[2] ☐ No	[3] ☐ Do not know

5.10) Other, please specify: ……

B6 Have you been diagnosed with melioidosis?

[1] ☐ No

[2] ☐ Yes, please specify year.....

If you cannot remember year, you can give rough estimate "6 months ago", "2 years ago"

[3] ☐ Do not know

Thank you for your time in providing information for this research project.

If you are a rice field owner,

please continue to next page; Section C: Rice field information.

participant code | Village's code | --|___|___|

Section C: Rice field information (only for rice field owner)

To be used as a baseline information and to understand associated factors related to presence of melioid-bacteria in your rice field (if found)

C1 In the past 12-month, how many rice farming were done in this rice field?

[1]☐ 1 time [2]☐ 2 times [3]☐ 3 times [4]☐ 4 times

C2 In the past 12-month, what kind of rice farming has been done?

2.1) "Nah-dum" (transplanting rice seedlings) [1]☐ Yes [2]☐ No [3]☐ Do not know

2.2) "Nah-whan" (direct seeding: broadcast seeds onto the soil surface) [1]☐ Yes [2]☐ No [3]☐ Do not know

2.3) "Nah-yod" (direct seeding: transplant rice seeds into holes on soil surface) [1]☐ Yes [2]☐ No [3]☐ Do not know

2.4) Others, please specify: ……..

……..

B3 What types of ploughing have been done in this rice field?

3.1) Used driving ploughing machine (sit on the machine) [1]☐ Yes [2]☐ No [3]☐ Do not know

3.2) Used pushing ploughing machine (walk with the machine) [1]☐ Yes [2]☐ No [3]☐ Do not know

3.3) Used animal ploughing e.g buffalo (walk with the animal) [1]☐ Yes [2]☐ No [3]☐ Do not know

3.4) Burn weeds which cover the rice field [1]☐ Yes [2]☐ No [3]☐ Do not know

3.5) Others, please specify: ……..

……..

C4 Type of fertilizers used in this rice field

4.1) Chemical fertilizer [1]☐ Yes [2]☐ No [3]☐ Do not know

4.2) Organic fertilizer made from plants [1]☐ Yes [2]☐ No [3]☐ Do not know

4.3) Organic fertilizer from animal wastes [1]☐ Yes [2]☐ No [3]☐ Do not know

4.4) Biological fertilizer [1]☐ Yes [2]☐ No [3]☐ Do not know

4.5) Others, please specify: ……

C5 Source of water for rice farming

5.1) Rain [1]☐ Yes [2]☐ No [3]☐ Do not know

5.2) Natural sources e.g. canal, river [1]☐ Yes [2]☐ No [3]☐ Do not know

5.3) Groundwater [1]☐ Yes [2]☐ No [3]☐ Do not know

5.4) Irrigation canals [1]☐ Yes [2]☐ No [3]☐ Do not know

5.6) Others, please specify: .....

C6 Apart from rice farming, has this piece of land been used for other type of farming?

[1]☐ No

[2]☐ Yes. Please specify: .....

C7 After harvesting season, how is this rice field managed?

[1]☐ Left it as it is until next planting season

[2]☐ Ploughing

[3]☐ Fertilizer spraying then ploughing

[4]☐ Ploughing then using green manure

[5]☐ Rice stubble and straw burning

[0]☐ Others, please specify: .....

C8 What is this rice field like when it is not used for rice farming or other farmings

[1]☐ Empty land without plants or weeds

[2]☐ Covered with other plants/ weeds/ trees at all times

[3]☐ Covered with other plants/ weeds/ trees sometimes

[4]☐ Flooded at all times

[5]☐ Other, please specify: .....

End of the questionnaire

Thank you for your time in providing information for this research project.

Appendix 2. A brochure for public about melioidosis developed and distributed by Bureau of Epidemiology, Ministry of Public Health, Thailand (in Thai)

มาทำความรู้จัก...โรคเมลิออยโดสิส

สาเหตุ

เกิดจากการติดเชื้อแบคทีเรีย เบอโคโดเลีย สูโดมาลิอาย (*Burkholderia pseudomallei*) ซึ่งเป็นเชื้อแบคทีเรียที่อาศัยอยู่ในดินและน้ำ พบได้ทุกภาคของประเทศไทย เชื้อเข้าสู่ร่างกายทำให้เกิดฝีหนอง หากเข้าสู่ปอดทำให้เกิดปอดอักเสบติดเชื้อ หรือเข้าสู่กระแสเลือด ทำให้เกิดภาวะติดเชื้อในกระแสเลือด การติดเชื้อมักรุนแรง นำไปสู่ภาวะ **หัวใจล้มเหลว ช็อก ระบบหายใจล้มเหลว และเสียชีวิต**

อาการ

เป็นโรคที่ไม่มีอาการแสดงจำเพาะ ผู้ป่วยส่วนใหญ่มักมีไข้สูง โดยพบว่าผู้ป่วยครึ่งหนึ่งมีอาการคล้าย**ปอดอักเสบติดเชื้อ** บางรายอาจมี **ไข้สูง ช็อก** จากการติดเชื้อในกระแสเลือดเพียงอย่างเดียว แต่บางรายอาจติดเชื้อในหลาย ๆ อวัยวะร่วมด้วย เช่น มีแผลติดเชื้อ มีฝีหนองที่ปอด ตับ หรือม้าม ส่วนใหญ่มีอาการรุนแรง รวดเร็ว แต่ผู้ป่วยบางรายอาจมีอาการเรื้อรังหรือไอเรื้อรัง คล้ายวัณโรคได้

การติดเชื้อ

การได้รับเชื้อจากสิ่งแวดล้อมโดยตรงผ่านทาง

1. การสัมผัสเชื้อในดินหรือน้ำ

เช่น การลงนา ทำนา ทำสวน การลุยน้ำ ดำน้ำ โดยไม่ใส่รองเท้าบูทหรืออุปกรณ์ป้องกันที่เหมาะสม เมื่อผิวหนังสัมผัสดินและน้ำเป็นเวลานาน เชื้อสามารถเข้าสู่ร่างกายได้โดยตรง โดยไม่จำเป็นต้องมีบาดแผล ถ้ามีบาดแผลเชื้อจะเข้าสู่ร่างกายได้ง่ายขึ้น

2. การกินอาหารหรือดื่มน้ำที่มีเชื้อปนเปื้อน

เช่น การดื่มน้ำบ่อ น้ำบาดาล น้ำประปาที่ไม่ได้ผ่านการฆ่าเชื้อด้วยคลอรีน น้ำดื่มที่ปนเปื้อนดินจากระบบท่อหรือการจัดเก็บ อาหารที่อาจปนเปื้อนดิน การใช้เครื่องกรองน้ำที่ไม่ได้มาตรฐาน หรือไม่ได้เปลี่ยนไส้กรองเป็นประจำ

3. การหายใจ

เช่น การอยู่ในที่เปิดโล่งหรือลงนาในขณะที่มีพายุฝน ลมกรรโชก หรือมรสุม สภาพอากาศเหล่านั้น สามารถพัดดินที่มีเชื้อขึ้นสู่อากาศ และเข้าสู่ร่างกายผ่านทางการหายใจได้

***การติดต่อจากคนสู่คนและจากสัตว์สู่คนพบได้น้อยมาก**

สถานการณ์โรคเมลิออยโดสิส

- ปี พ.ศ. 2555 พบผู้ป่วยวินิจฉัยด้วยการเพาะเชื้อยืนยันทั่วประเทศ **1,735 ราย เสียชีวิต 696 ราย***
- สำนักระบาดวิทยา ได้รับการรายงาน**ผู้ป่วยเสียชีวิต จำนวน 13 ราย** ในปี พ.ศ. 2555 และ**เพิ่มขึ้นเป็น 233 ราย ในปี พ.ศ. 2560 ****
- พบผู้ป่วยได้ทุกวัย ตั้งแต่แรกเกิด ผู้ป่วยส่วนใหญ่**อายุมากกว่า 45 ปี** และมีโรคประจำตัว เช่น โรคเบาหวาน
- พบผู้ป่วยได้ทุกภาค โดย**พบมากในภาคตะวันออกเฉียงเหนือ ภาคตะวันออก และภาคใต้**
- พบผู้ป่วยได้ตลอดทั้งปี พบจำนวน**ผู้ป่วยจะสูงขึ้นในช่วงฤดูฝน** เนื่องจากเป็นช่วงฤดูทำนา มีการลงนาสัมผัสดินและน้ำมากกว่าปกติ และมีลมมรสุม ทำให้เชื้อปนเปื้อนในอากาศได้

* ข้อมูลจากการศึกษาร่วมกันระหว่างสำนักระบาดวิทยาและหน่วยวิจัยมหิดลอ๊อกซ์ฟอร์ดในโรงพยาบาลศูนย์และโรงพยาบาลทั่วไปทั้งประเทศ ในปี พ.ศ. 2555

** ข้อมูลจากระบบเฝ้าระวังทางระบาดวิทยา สำนักระบาดวิทยา ปี พ.ศ. 2560 จากการรายงานผู้ป่วยวินิจฉัยด้วยการเพาะเชื้อยืนยันจากโรงพยาบาลศูนย์และโรงพยาบาลทั่วไปอย่างน้อย 5 แห่ง

การวินิจฉัยโรคเมลิออยโดสิสสำหรับสถานพยาบาล

การวินิจฉัยโรคอย่างถูกต้อง

มีผลต่อวิธีให้การรักษาและการรอดชีวิตของผู้ป่วย ควรใช้การตรวจด้วยวิธีเพาะเชื้อเท่านั้น เนื่องจากการตรวจแอนติบอดีต่อเชื้อเมลิออยโดสิสด้วยวิธี Indirect hemagglutination (IHA) นั้นมีความไวต่ำ ความจำเพาะต่ำ ส่วนใหญ่ได้ผลบวกปลอม เพราะคนไทยส่วนใหญ่เคยสัมผัสดินและน้ำ (โดยเฉพาะอย่างยิ่งเกษตรกร) จึงมีแอนติบอดีต่อเชื้อนี้ประมาณร้อยละ 10 - 30 ทำให้ผลบวกจากการตรวจ IHA ในผู้ป่วยที่มีไข้ หรือผู้ป่วยปอดอักเสบทั่วไปมักเป็นผลบวกลวง

การเพาะเชื้อ พบเชื้อ *Burkholderia pseudomallei*

ไม่ว่าจากสิ่งส่งตรวจใดถือเป็นการตรวจยืนยันว่าเป็นโรคเมลิออยโดสิส เนื่องจากเชื้อนี้ไม่ใช่เชื้อที่พบได้ในร่างกายคนปกติ และการพบเชื้อในปัสสาวะแม้เพียงตัวเดียว ก็ยืนยันการติดเชื้อได้

หลักสำคัญในการรายงานผลทางห้องปฏิบัติการจุลชีววิทยา

การเพาะเชื้อยังมีข้อจำกัดที่ต้องอาศัยห้องปฏิบัติการจุลชีววิทยาคลินิก และบุคคลที่มีความรู้ความชำนาญในการระบุเชื้อ อีกทั้งยังต้องใช้เวลาในการตรวจเฉลี่ย 3 - 7 วัน

- ต้องแน่ใจว่าเชื้อที่เป็นแกรมลบรูปร่างแท่งและผลทดสอบอ๊อกซิเดสเป็นบวก (Gram-negative bacilli, oxidase-test positive) ไม่ใช่เชื้อ *Burkholderia pseudomallei* โดยเฉพาะอย่างยิ่งเชื้อที่เพาะได้จากเลือด
- เชื้อที่มีลักษณะโคโลนีคล้ายเชื้อที่แยกจากดิน ไม่ควรระบุว่าเป็นเชื้อปนเปื้อน หรือรายงานว่าเป็นเชื้อ *Pseudomonas* spp. โดยที่ไม่ได้ตรวจสอบว่าเป็นเชื้อ *Burkholderia pseudomallei* หรือไม่
- ถ้าผลการทดสอบความไวต่อยาของเชื้อแกรมลบ พบว่าเชื้อไวต่อยา Ceftazidime, Amoxicillin-clavulanic acid และดื้อต่อยา Gentamycin และ Colistin ควรนึกถึงเชื้อ *B.pseudomallei* มากขึ้น
- ผู้ป่วยทุกรายที่ มีผลเพาะเชื้อพบเชื้อชนิดนี้ ต้องรายงานเข้ามาในระบบเฝ้าระวังโรค (รง. 506)

3 มาตรการหลักในการป้องกันโรคเมลิออยโดสิส

ดื่มน้ำต้มสุกเสมอ

เนื่องจาก น้ำฝน น้ำบ่อ น้ำบาดาล น้ำประปาตามท่อ อาจมีเชื้อเมลิออยโดสิสปนเปื้อนได้

สวมรองเท้าบูทยาว

หรือชุดลุยน้ำ ถุงมือยาว เวลาลงนาเสมอ หลีกเลี่ยงการสัมผัสดิน น้ำโดยตรง ไม่เดินเท้าเปล่า และสวมเครื่องป้องกันที่เหมาะสมเวลาที่ต้องสัมผัสดิน น้ำ เช่น จับปลา ลุยน้ำ ลุยโคลน

หลีกเลี่ยงอยู่ที่โล่งแจ้ง

หรือลงนาในขณะที่มีพายุฝน ลมมรสุมหรืออากาศแปรปรวน

ร่วมกันทำ ป้องกันได้

คำถามที่พบบ่อย

1) โรคเมลิออยโดสิสเป็นโรคใหม่ ?

คำตอบ: ไม่ใช่ โรคเมลิออยโดสิสเป็นโรคติดเชื้อประจำถิ่นของทุกประเทศในภูมิภาคเอเชียตะวันออกเฉียงใต้ พบครั้งแรกในประเทศพม่าเมื่อ 100 ปีก่อน ส่วนการพบครั้งแรกในประเทศไทย พบเมื่อ 50 ปีที่ผ่านมา

2) ทำไมไม่เคยได้ยินชื่อโรคเมลิออยโดสิสมาก่อนเลย ?

คำตอบ: เนื่องจากโรคนี้ยากต่อการวินิจฉัย ผู้ป่วยส่วนใหญ่เสียชีวิตก่อนที่ผลเพาะเชื้อยืนยันจะถูกรายงานกลับมาที่แพทย์ ผู้ป่วยโรคเมลิออยโดสิสส่วนใหญ่ที่เสียชีวิตมักได้รับการบอกว่าเป็นโรคติดเชื้อแบคทีเรีย หรือโรคติดเชื้อในกระแสเลือด ปัจจุบันมีการกระตุ้นให้เกิดการรายงานผู้ป่วยที่มีผลเพาะเชื้อยืนยันในระบบเฝ้าระวังโรคให้ครบถ้วน เพื่อให้เกิดการรณรงค์เกี่ยวกับการวินิจฉัย การรักษา และการป้องกันโรคเมลิออยโดสิส

3) อาการอย่างไร ควรรีบไปโรงพยาบาล ?

คำตอบ: ผู้ป่วยไม่จำเป็นต้องตื่นตระหนกเกี่ยวกับโรคเมลิออยโดสิส เมื่อรู้สึกไม่สบาย ไข้สูง จำเป็นต้องไปสถานพยาบาลตามปกติ แพทย์ในสถานพยาบาลสามารถให้การวินิจฉัยและให้การรักษาที่เหมาะสมได้

4) สวมรองเท้าบูทลงนา ทำไมไม่ได้หรอก เดินไม่ได้ ทำยังไง ?

คำตอบ: บูทยาว และชุดลุยน้ำ สามารถใส่ทำงานในนาได้ดี ชาวนาควรทาแป้งและสวมถุงเท้ายาวก่อนสวมบูท จะช่วยให้ใส่บูทได้นานขึ้น ไม่เสียดสี และไม่ควรลงนาเท้าเปล่าเพราะอาจติดเชื้อโรคเมลิออยโดสิสและโรคอื่นๆ ได้ อีกทั้งอาจเกิดบาดแผลที่เท้าได้ง่ายขึ้น โดยเฉพาะผู้ป่วยเบาหวาน อาจติดเชื้อได้ง่าย รายละเอียดเกี่ยวกับการใส่บูทลงนาและการป้องกันโรคสามารถอ่านเพิ่มเติม ได้ที่ https://melioidosisubon.wixsite.com/premel และ www.melioidosis.info

5) ทุกคนควรทำอย่างไรเพื่อให้จำนวนผู้เสียชีวิตจากโรคเมลิออยโดสิสน้อยลง ?

คำตอบ: ประชาชนทั่วไป ควรป้องกันตัวเองจากการติดเชื้อ ตามคำแนะนำ โดยเฉพาะอย่างยิ่งสวมรองเท้าบูทยาวทุกครั้งเวลาลงนา สถานพยาบาลควรให้ข้อมูลผู้ป่วยและญาติผู้ป่วยที่เป็นโรคเมลิออยโดสิสที่มีผลเพาะเชื้อยืนยันและรายงานผู้ป่วยพร้อมผลการรักษา ตามระบบเฝ้าระวังโรคทางระบาดวิทยาอย่างครบถ้วน

สายด่วนกรมควบคุมโรค 1422

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